BIOFILM REACTORS FOR INTENSIVE PRODUCTION OF BICARBOXYLIC ACIDS

Mariateresa Ferone

Dottorato in Biotecnologie – XXX ciclo

Università degli studi di Napoli Federico II

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Dottoranda: Mariateresa Ferone
Relatore: Prof. Antonio Marzocchella
Prof. Piero Salatino
Prof. Giuseppe Olivieri

Coordinatore: Prof. Giovanni Sannia

Scegli il lavoro che ami
e non lavorerai neppure un giorno in tutta la tua vita
-Confucio

A scientist in his laboratory is not a mere technician:
he is also a child confronting natural phenomena
that impress him as though they were fairy tales
-Marie Curie

Para hacer las cosas bien es necesario:
primero, el amor, segundo, la técnica
-Antoni Gaudì

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RIASSUNTO

Per molti anni il petrolio è stato utilizzato sia come fonte di combustibili sia come fonte per la produzione di *building block* per prodotti di varia natura. Attualmente, la domanda annua media di petrolio utilizzata per la produzione di prodotti chimici è superiore a un miliardo di barili e il consumo complessivo di combustibili liquidi come petrolio greggio e gas naturale ammonta a oltre 30 miliardi di barili all'anno.

Negli ultimi anni la ricerca scientifica ed industriale ha cercato strade alternative alla via petrolchimica per la produzione di prodotti chimici e in molti casi gli sforzi si sono concretizzate dando vita alle “bioraffinerie”. Con il termine bioraffineria si indicano i processi volti alla produzione sia di prodotti con valenza economica quali mangimi, combustibili, prodotti chimici, polimeri, lubrificanti, adesivi, fertilizzanti, sia di servizi (e.g. bioenergia) utilizzando come materie prime le biomasse. Questo concetto sta emergendo come strategia per ridurre l’emissione di biossido di carbonio nell’atmosfera, l’utilizzo di petrolio greggio e la dipendenza da fonti non rinnovabili di energia.

L'approccio petrochimico per la produzione di prodotti e materiali è attualmente più competitivo rispetto all'approccio biologico grazie alla continua ottimizzazione e ricerca durata per più cinquant’anni. La produzione *biobased* di prodotti chimici non è ancora competitiva perché è la meno sviluppata ed è la più complicata di tutte le operazioni di *biorefinery*. Tuttavia, questa tendenza cambia presto a causa dell'esaurimento dei combustibili fossili e dell'ottimizzazione dei processi biologici. Un ampio portafoglio di sostanze chimiche tradizionalmente prodotte per la via petrolchimica possono essere oggigiorno prodotti via processi biotecnologici a partire da risorse rinnovabili (e.g. biomasse) mediante microrganismi come biocatalizzatori (Ragauskas, 2006).

Gli acidi organici sono tra i primi prodotti biotecnologici prodotti. Gli acidi organici sono tradizionalmente utilizzati nell'industria dell’alimentazione umana e animale (Cherringtona et al., 1991). Grazie al loro gruppi acilici, sono estremamente utili come elementi di partenza per l'industria chimica. Infatti, i chetoacidi o gli idrossiacidi costituiscono ottimi *building block* chimici per la produzione di poliesteri, mentre gli acidi bicarbossilici possono essere utilizzati per produrre poliammidi (Becker et al., 2015).

L'acido succinico (AS), un acido bicarbossilico alifatico a quattro atomi di carbonio, è stato riconosciuto dal Dipartimento dell'Energia statunitense come uno dei 12 prodotti chimici ad alto valore aggiunto che possono essere prodotti dalla biomasse rinnovabili nel 2004 (Werpy and Petersen, 2004). La produzione di acido succinico bio-based può potenzialmente sostituire il processo di produzione petrolchimico, con notevoli benefici ambientali: 1) l'uso di sostanze rinnovabili come substrato per la fermentazione (Salvachúa et al., 2016b) e 2) l’utilizzo di CO_2, che è fissata in acido succinico durante la fermentazione (Van der Werf et al., 1997). Riconoscendo la sua potenziale importanza, una vasta ricerca è stata condotta a livello globale per rendere più competitivo il processo la produzione di acido succinico bio-based.

L’acido succinico trova applicazioni in diversi settori industriali: è utilizzato nell’industria alimentare, nell’industria farmaceutica e cosmetica e per la produzione di detergenti, surfattanti e agenti anti-corrosivi (Zeikus et al., 1999). L’utilizzo dell’acido succinico in tali settori lo rendono un prodotto a elevato valore aggiunto e,

di conseguenza, la sua produzione non richiede materie prime particolarmente economiche. Invece, l'uso dell'acido succinico come *chemical building block* richiede che i costi di produzione, materie prime incluse, siano molto bassi. La produzione dell'acido per via biotecnologica potrebbe portare a una riduzione dei costi di produzione grazie alle elevate rese di conversione, alla riduzione delle emissioni di gas serra e agli altri vantaggi per l'ambiente sopra menzionati.
I costi e l'impatto ambientale di un processo *bio-based* per la produzione di acido succinico dipendono fortemente da quattro fattori: 1) la materia prima selezionata e il tipo di pretrattamento necessario per produrre un mezzo di fermentazione completo; 2) il microrganismo selezionato per la fermentazione; 3) il tipo di bioreattore impiegato; 4) il processo di recupero e purificazione dell'acido succinico.
Per quanto riguarda il primo punto, potenziali materie prime per la produzione di acido succinico per via fermentativa sono le biomasse lignocellulosiche e i residui industriali a contenuti zuccherini semplici o complessi (Akhtar et al., 2014; Wan et al., 2008). Le biomasse lignocellulosiche consistono prevalentemente di cellulosa, emicellulosa e lignina che formano una struttura complessa e richiedono specifici pretrattamenti e idrolisi enzimatica per poter rilasciare zuccheri fermentabili. Alcune tipologie di sottoprodotti industriali, invece, (es. siero di latte) possono essere utilizzati dai microorganismi senza alcun pretrattamento.
Con riferimento alla tipologia reattoristica utilizzata, tipicamente la produzione viene effettuata in fermentatori eserciti in modalità batch, caratterizzati da tempi morti lunghi. In diverse *review* è stato evidenziato il potenziale dei reattori a biofilm per la produzione intensiva di prodotti chimici (Qureshi et al., 2005). I vantaggi principali dei reattori a biofilm sono: possibilità di esercire portate elevate, concentrazioni cellulare elevate e possibilità di operare il reattore per lunghi tempi.
Actinobacillus succinogenes è tra i ceppi batterici *wild type* più promettenti in quanto è in grado di utilizzare come substrato per la fermentazione diversi zuccheri. Esso è caratterizzato da un'elevata resa di fermentazione ed è classificato come microrganismo di livello di biosicurezza 1 da DSMZ e ATCC.

Scopo della tesi. Lo scopo del lavoro svolto durante il DdR è stata lo studio del processo di produzione di acido succinico per fermentazione di zuccheri da biomasse e/o da residui dell'industria agro-alimentare. Il lavoro è stato svolto presso il Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale dell'Università degli Studi di Napoli Federico II.
Le attività sono state articolate in due linee: i) la caratterizzazione del processo di produzione dell'acido succinico - con riferimento alla concentrazione e rese - utilizzando per la fermentazione sia substrati rinnovabili e sia residui zuccherini; ii) lo sviluppo di un reattore a biofilm esercito in continuo per la produzione intensiva di acido succinico.
Caratterizzazione del processo di produzione dell'acido succinico con riferimento a concentrazione e rese utilizzando
Lo studio è stato mirato alla valutazione della prestazione di fermentazione, in termini di concentrazione e resa dell'acido succinico prodotti, utilizzando due diversi tipi di substrati: 1) zuccheri tipicamente presenti negli idrolizzati lignocellulosici (glucosio, mannosio, arabinosio e xilosio, GMAX); 2) bevande ad alto contenuto di zuccheri (*high sugar content beverage*, HSCB).

L'attività al punto 1) è stata focalizzata a valutare la capacità del batterio *A. succinogenes* a fermentare gli zuccheri tipicamente presenti nella frazione idrolizzata di biomasse lignocellulosiche: glucosio, mannosio, arabinosio e xylosio (GMAX) (Ezeji and Blaschek, 2008). I test di fermentazione batch sono stati effettuati con un terreno di coltura standard contenente gli zuccheri prima citati. Il processo di conversione è stato caratterizzato in termini di produzione di metaboliti (acido succinico, acido formico e acido acetico), conversione dello zucchero, concentrazione cellulare e pH. Lo studio ha incluso sia test utilizzando zuccheri singoli, che test con una miscela di zuccheri, la cui composizione mima quella di un idrolizzato lignocellulosico, per valutare i possibili effetti sinergici/interferenti. I test batch sono stati focalizzati sulla caratterizzazione preliminare della fermentazione con l'obiettivo di evidenziare le caratteristiche rilevanti del processo, quali concentrazione e resa di acido succinico.
Le indagini hanno evidenziato che i) *A. succinogenes* è il grado di utilizzare tutti gli zuccheri investigati per produrre acido succinico; ii) la velocità di crescita di *A. succinogenes* diminuisce con la concentrazioni di glucosio, mentre per gli altri zuccheri – mannosio, xilosio, arabinosio- non si osserva tale fenomeno; iii) la presenza dei quattro zuccheri in miscela ha portato a un incremento della concentrazione di acido succinico prodotto rispetto a quella attesa (effetto sinergico) e un aumento della selettività di acido succinico rispetto agli altri acidi prodotti (acido acetico e formico).
L'attività al punto 2) ha riguardato sottoprodotti dell'industria delle bevande. Esse possono spesso rappresentare un rifiuto dell'industria alimentare, acque di lavaggio, sequestro di beni non correttamente smaltiti o illegali, prodotti che hanno superato la data di scadenza. Gli zuccheri tipicamente presenti in tali bevande sono glucosio, fruttosio e saccarosio. Sono stati effettuati esperimenti di fermentazione batch utilizzando sia le bevande tal quali, ovvero senza l'aggiunta di nessun fonte di azoto o altri elementi, sia bevande addizionate con estratto di lievito e sali minerali. Inoltre, alcuni esperimenti sono stati effettuati sottoponendo le bevande a un pretrattamento di idrolisi mediante acido cloridrico in autoclave, in modo da ottenere la completa idrolisi del saccarosio nei suoi monomeri – glucosio e fruttosio.
I test di fermentazione batch hanno evidenziato che *A. succinogenes* è in grado di crescere su tutti gli HSCBs addizionate con estratto di lievito, ma anche su succhi di frutta non addizionati. Lo step di idrolisi acida delle bevande ha migliorato le prestazioni della fermentazione in termini di produzione di acido succinico e conversione totale degli zuccheri.
Queste attività hanno evidenziato che *A. succinogenes* è un microrganismo promettente per la produzione su scala industriale di acido succinico un substrato economico e abbonante come le biomasse lignocellulosiche o gli scarti dell'industria delle bevande.

Sviluppo di un reattore continuo a biofilm per la produzione intensiva di acido succinico

Lo sviluppo e l'ottimizzazione di un reattore a biofilm che operi in continuo per la produzione di acido succinico richiede la conoscenza della cinetica di crescita e di produzione dell'acido da parte di *A. succinogenes.* Infatti, il problema principale per la produzione di acidi organici mediante fermentazione a scala industriale, come ad esempio. acido citrico e acido lattico - è il l'effetto di inibizione dell'acido prodotto

sulla crescita cellulare (Nandasana and Kumar, 2008). In letteratura sono presenti alcuni lavori che propongono modelli cinetici per la crescita di *A. succinogenes* e la produzione di acido succinico utilizzando diversi substrati, ma questi modelli sono stati ottenuti in modalità batch. Per poter progettare, ottimizzare e controllare in maniera più affidabile un reattore a biofilm continuo, è necessario avere una caratterizzazione cinetica completa ottenuta operando il fermentatore in continuo.
Le cinetiche sono state stimate elaborando i risultati di test di fermentazione condotti in continuo in reattori a perfetta miscelazione (*continuous stirred tank reactor*, CSTR) a pH controllato al valore ottimale (6.8) e utilizzando glucosio come fonte di carbonio.
Per ciascuno stato stazionario, il processo di fermentazione è stato caratterizzato in termini di concentrazione cellulare, concentrazione degli acidi prodotti e del glucosio consumato, in un ampio intervallo di condizioni di esercizio. Alcuni test sono stati effettuati aggiungendo all'alimentazione l'acido acetico e formico (prodotti di fermentazione) ad elevata concentrazione. L'intento è stato quello di pervenire alla stima dei parametri cinetici caratteristici del comportamento inibitorio che tali acidi hanno sulla crescita del microorganismo. I risultati sperimentali sono stati interpretati attraverso il seguente modello cinetico, che prevede inibizione combinata dovuta a più prodotti:

$$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i} \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \left(1 - \frac{FA}{FA_{max}}\right)^{n_{FA}}$$

La regressione dei dati sperimentali in accordo al modello cinetico proposto hanno permesso di pervenire alla stima dei seguenti parametri cinetici:

μ_{max}= 1.43 h^{-1}

K_G= 6.96 g/L

K_{SA}= 4.10 g/L

AA_{max}= 80 g/L

n_{AA}= 0.1

FA_{max}= 45.6 g/L

n_{FA}= 0.53

Nelle condizioni di esercizio investigate, i risultati hanno evidenziato che non si è osservata inibizione da substrato e che l'acido acetico non contribuisce in maniera significativa all' inibizione della crescita cellulare e della produzione di acido succinico.
L'attività mirata alla produzione intensiva di acido succinico ha riguardato la progettazione, la messa a punto, l'esercizio e l'ottimizzazione di un reattore a biofilm a letto fisso (*packed bed biofilm reactor*, PBBR). Il reattore è costituito da un vessel cilindrico di 8 cm di altezza e 5 cm di diametro interno e sono stati utilizzati degli anelli di Tygon di 3mm come supporto per la crescita del biofilm. È stato studiato l'effetto delle condizioni di esercizio investigate - velocità di diluizione (*dilution rate*, D), composizioni del terreno - sulle prestazioni del reattore. Gli esperimenti sono stati effettuati alimentando il reattore con soluzioni contenenti un ampio spettro di fonti di carbonio, come riportato di seguito:

- Terreno sintetico contenente glucosio come fonte di carbonio. D fissato tra 0,5 h^{-1} and 2,4 h^{-1};
- Terreno sintetico contenente glucosio e xilosio a frazione fissata tra 0 e 1. D fissato a 1.24 h^{-1};
- Terreno sintetico contenente xilosio come fonte di carbonio. D fissato tra 0.5 h^{-1} to 1.44 h^{-1};
- Terreno sintetico contenente glucosio, arabinosio e xilosio. D fissato tra 0.7 h^{-1} to 1.44 h^{-1}.

È stato studiato l'effetto di idrossimetilfurfurale (*hydroxymethylfurfural*, HMF) e furfurale sulle prestazioni del bioreattore. Queste specie sono tra i principali inibitori della fermentazione e sono tipicamente presenti negli idrolizzati lignocellulosici.
I test condotti evidenziano che la concentrazione di acido succinico prodotto diminuisce all'aumentare della velocità di diluizione. Particolarmente interessante è la produttività volumetrica di acido succinico massima raggiunta: 35.0 g/L*h, misurata a D= 1.9 h^{-1} è la più alta riscontrabile in letteratura. Risultati ottimali sono stati ottenuti alla velocità di diluizione di 0.5 h^{-1}: 43 g/L di acido succinico, grado di conversione del substrato di 0.88 e una produttività volumetrica di 22 g/L*h.
L'attività svolta ha evidenziato come sia possibile ottenere un miglioramento delle prestazioni di fermentazione passando dalla modalità batch a quella continua e utilizzando un reattore a cellule immobilizzate.

Produzione di acido adipico bio-based presso la Chalmers University of Technology

Le attività svolte durante il periodo di ricerca presso la divisione di Biotecnologie Industriali della Chalmers University of Technology (Goteborg, Svezia) sono state finalizzate allo sviluppo di una *microbial cell factory* per la produzione di acido adipico da risorse rinnovabili. Le attività sono state articolate in due linee: i) identificazione e caratterizzazione *in vitro* di un enzima con attività di ossidoreduttasi (nativo o ingegnerizzato) in grado di catalizzare il secondo step enzimatico del pathway proposto per la produzione di acido adipico; ii) studio della fisiologia e della robustezza di due ceppi di lievito in presenza di acido adipico al fine di valutare la tolleranza dei due ceppi a tale acido.
Per le attività al punto 1) sono state selezionate due fumarato reduttasi, una del lievito *Saccharomyces cerevisiae* (Frd1) e una del batterio *Shawanella oneodensis* (SO_0970). Entrambi gli enzimi sono stati espressi in *E. coli* e purificati ed è stato messo a punto un saggio anaerobico per misurarne l'attività enzimatica. Inoltre in entrambi gli enzimi sono stati introdotte sostituzioni amminoacidiche per ampliarne il sito attivo e permettere la catalisi sui substrati target.
Le attività al punto 2) hanno riguardato lo studio della tolleranza all'acido adipico di due lieviti: *Saccharomyces cerevisiae* (noto anche come organismo modello) e *Candida viswanathii*; quest'ultimo è stato scelto perché da uno studio preliminare è risultato altamente tollerante alla presenza di acido adipico. A tale scopo sono stati condotti test sistematici di crescita in presenza di acido adipico in bioreattori su scala di laboratorio.
Tali attività sono descritte in dettagli nella sezione Appendix.

SUMMARY

The study carried out during the present Ph.D. program aimed at investigating succinic acid production by fermentation. The work was carried out at the Dipartimento di Ingegneria Chimica, dei Materiale e della Produzione Industriale of the Università degli Studi di Napoli 'Federico II'. The commercial strain *Actinobacillus succinogenes* DSM 792 was selected for its ability to produce succinic acid at high concentration and yield from a broad variety of carbon source. The activities were articulated along the two paths described hereinafter.

Characterization of the succinic acid fermentation process as regards the concentration and yields

The study was aimed at the assessment of the fermentation performance - in terms of succinic acid concentration and yield – with reference to two classes of feedstocks: 1) sugars typically present in lignocellulosic hydrolysates (glucose, mannose, arabinose and xylose); 2) high sugar content beverages (HSCBs).

The investigations were carried out with batch fermentation of free *A. succinogenes* cells at different sugar concentration. The conversion process was characterized in terms of metabolite production (succinic acid, formic acid and acetic acid), sugar conversion, pH and cell concentration. The study included tests with single sugar and sugar mixtures (synthetic and commercial): i) glucose, mannose, arabinose and xylose (GMAX) to mime the composition of a lignocellulosic hydrolysate; ii) sucrose, glucose and fructose (SGF), the sugars typically present in leftover beverages.

All the investigated sugars were converted in succinic acid by *A. succinogenes*. Tests with mixtures pointed out that the fermentation took advantage of the co-presence of different sugars with respect to the tests carried out with single sugars. The synergy effect of the sugars led to an increase in final succinic acid concentration and SA selectivity.

Development of a continuous process for the succinic acid production

Continuous fermentation tests carried out in a CSTR were aimed to the systematic characterization of the kinetics of cell growth and succinic acid production. The continuous fermentation were carried out by feeding the CSTR with a glucose bearing medium at a wide interval of dilution rate.

The kinetics of *A. succinogenes* growth on glucose was investigated by assessing the effects of glucose and acids - succinic, acetic, and formic - on cell growth. The experimental results were successfully correlated by a multiple product-inhibited interacted growth model. Experimental results pointed out the individual inhibitory effect of the acids on *A. succinogenes* growth. Under the investigated operating conditions, results pointed out that: no substrate inhibition was observed; acetic acid did not inhibit the cell growth and the succinic acid production.

The activity aimed at the intensive succinic acid production regarded the design, set-up, operation and optimisation of a packed bed biofilm reactor (PBBR). The effects of the operating conditions - dilution rate (D) and medium compositions (glucose, GX, xylose, GAX) - on the PBBR performances were investigated. Succinic acid concentration, productivity and sugar(s) conversion generally decreased with D. A maximum succinic acid productivity of 35.0 g/L*h was achieved at a D= 1.9 h^{-1}. The effect of HMF and furfural, the main inhibitors found in lignocellulosic hydrolysate, on succinic acid production was also investigated. HMF resulted to have a higher inhibition effect on succinic acid production compared to furfural.

1. INTRODUCTION

1.1 Organic acids: market and application

Organic acids are among the first biotechnological good produced. Organic acids are traditionally used in the food and feed industry (Cherringtona et al., 1991). However, their functional groups make them extremely useful as starting building blocks for the chemical industry. Indeed, keto- acids or hydroxy-acids are good building blocks for polyesters while bicarboxylic acids can be used to produce polyamides (Becker et al., 2015). Of the twelve sugar-derived building-block chemicals identified by the US Department of Energy, nine are organic acids (Werpy and Petersen, 2004).

Most of the organic acids can be produced by microbial processes: they are the end-product of the microbial metabolism or the least natural intermediates in the main metabolic pathways. Table 1.1 summarize the organic acid currently produced, their applications and the fraction produced through a bio-based process.

Table 1.1*: Industrially relevant organic acids and their main applications (Sauer et al., 2008).*

Number of C atoms	Organic acid	Annual production (ktons)	Annual production by microbial process (ktons)	Application
C2	Acetic acid	12 100	1200	Vinyl and cellulose acetate for polymers, ethylacetate as green solvent
	Oxacil acid	190	-	Bleaching agent, synthetic intermediate, complexing agent
C3	Acrylic acid	5 750	2 900	Polymers production
	3-hydroxypropionic acid	n.a.	-	Production of biodegradable polymers
	Lactic acid	500	500	Food and beverage, biodegradable polymers production
	Propionic acid	450	n.a	Food and feed
C4	Butyric acid	50	n.a.	Feed, therapeutics, aroma, fragrance
	Fumaric acid	23	-	Food and feed, polyesters resins
	Malyc acid	10	-	Potential to replace maleic anhydride
	Succinic acid	47.5	38	Potential to replace maleic anhydride, manufacture of tetrahydrofuran, polymers
C5	Itaconic acid	15	15	Specialty monomer
	Levulinic acid	450	-	Possible precursor of bulk chemicals
C6	Adipic acid	3 000	-	Production of nylon 6,6, ester used as plasticizers and lubricants
	Ascorbic acid	80	-	Food additive
	Citric acid	1 600	1 600	Food additive
	Glucaric acid	n.a.	-	Production of nylon, new building-block
	Gluconic acid	87	87	Food additive, metal chelator

n.a states for no data are available

"-"indicates that these acids are not microbially produced on an industrial level to the author knowledge.

1.2 Succinic acid

Succinic acid (SA), also known as amber acid or butanedioic acid, is a four -carbon aliphatic bicarboxylic acid. It is a colourless crystal, water-soluble and with an acid-taste; it was first discovered by Georgeus Agricola in 1946 during dry distillation of

amber. The US Department of Energy included succinic acid among the 12 top value added chemicals that can be produced from sugars (Werpy and Petersen, 2004). Since the 1942 action of the US-DoE, extensive research has been carried out on the fermentative production of this molecule, which led to the development of several cost-effective processes for fermentative SA production from renewable resources.

Figure 1.1, reports the main existing market sectors for succinic acid are the chemical, the food and the pharmaceutical. The chemicals market includes: surfactant, detergent, extender and foaming agent, and ion chelator (to prevent corrosion and pitting of metals). The food market includes: acidulant and pH modifiers, flavouring agent, anti-microbial agents. The health-related agents market include: cosmetic, pharmaceuticals, antibiotics, amino acids, and vitamins (Zeikus et al., 1999). Resins, coatings, dyes, and inks are the largest application segment accounting for over 9.0 kilo tons of the global market in 2014. Pharmaceuticals were the second largest application segment valued at USD 14.9 million in 2014.

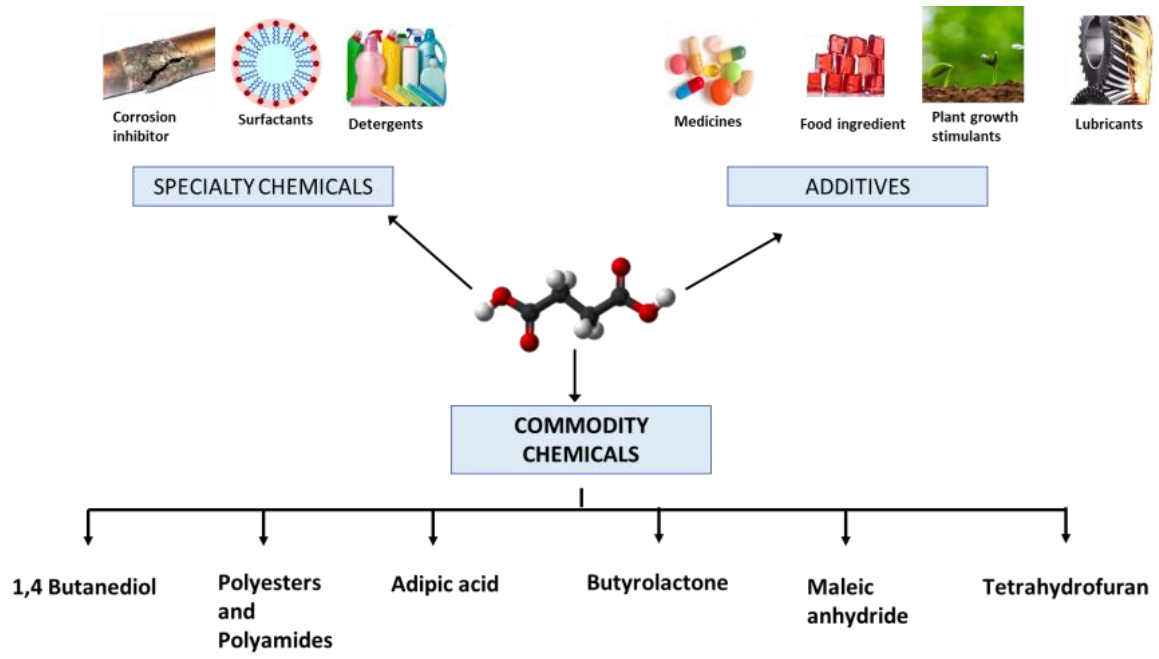

***Figure 1.1**: Overview of applications for succinic acid and products derived from it.*

A much large market of SA is expected as a precursor for numerous industrially valuable chemicals. In fact, the structure of the succinic acid - linear saturated bicarboxylic acid – makes it an intermediate building block for the production of a number of industrially important chemicals, including adipic acid (a precursor for Nylon x,6), 1,4-butanediol (1,4-BDO; a precursor for polyesters and Spandex), tetrahydrofuran (THF; an important solvent and a precursor for poly[tetramethylene ether] glycol), N-methylpyrrolidone (NMP; an important solvent in chemical and lithium-ion battery industries), 2-pyrrolidone (a precursor for pharmaceuticals and vinylpyrrolidone), gamma-butyrolactone (GBL; a precursor for pesticides, herbicides, and pharmaceuticals), and other green solvents and chemicals. In addition, the increasing demand for succinic acid is expected because of its use in the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides (Nylon®x,4) (Willke and Vorlop, 2004).

Global succinic acid market size was estimated at 47.5 kilo tons in 2014 and it is projected to reach 251 kilotons by 2021, registering a CAGR of 27.2%. The global succinic acid market is projected to reach USD 701 Million at a CAGR of 28.0%. This growth is fuelled by the increasing production capacities, rising development strategies, growing shift toward bio-based renewable substitutes in the plastics industry, and growing application sectors globally (Bio-based News, 2015).

The annual production capacity of bio-based succinic acid in the period 2013–2014 was around 38,000 tonnes, that constitutes 49% of the total market. The bio-based succinic acid market is expected to reach 600,000 t by 2020 with a projected market size of \$539 × 10^6. he major drivers for this growth will be volatility in fossil fuel prices, rise in carbon footprints, and an interest in using locally available raw materials. Furthermore, increasing global demand of green chemicals will trigger its adoption in wide range of applications, especially, in bio plastics, making it a strong platform chemical.

To make bio-based succinic acid a competitive platform chemical, its production cost should be around 1 \$/kg of acid. The current market price for biobased succinic acid is around 2.94 \$/kg, while the price for fossil-based SA is around 2.5 \$/kg. Once a competitive microbial production process for SA is established the market for this bio-based product will increase (Taylor et al., 2015).

1.3 Conventional VS biotechnological SA production route

Historically, succinic acid was obtained from amber by distillation and it has been known as spirit of amber (Boy and Lappe, 2000). Today, the conventional processes for the synthesis of succinic acid starts from fossil resources (Figure 1.2) and they are based on catalytic hydrogenation of 1,4-dicarboxylic unsaturated C4 acids or anhydrides, and in a lesser extent via oxidation of 1,4-butanediol. Carbonylation processes starting from ethylene glycol, ethylene, acetylene, dioxane or unsaturated C3 carboxylic acids or propiolactone are also paths to produce succinic acid or its esters. Most direct petrochemical route to succinic acid is liquid-phase maleic anhydride hydrogenation to succinic anhydride (SAN) followed by the hydration of the later to succinic acid.

The catalytic hydrogenation technology is a consolidated industrial process: succinic acid is produced at high yield and purity, but the process expensive and can cause serious environmental problems (Cok et al., 2013).

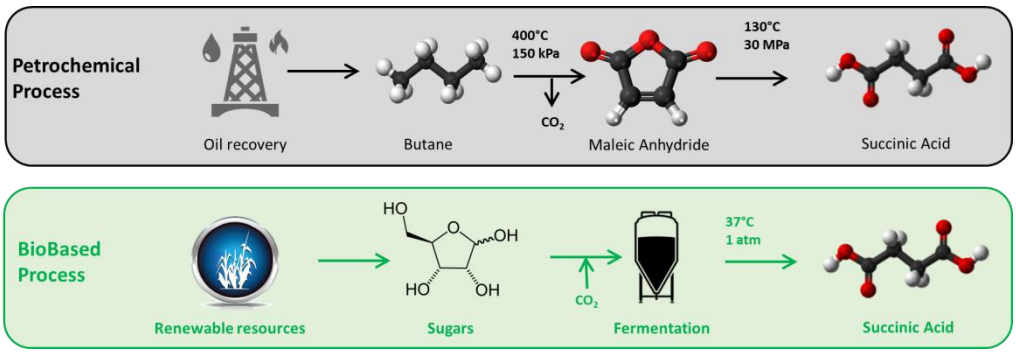

***Figure 1.2**: Conventional vs biotechnological route to produce succinic acid.*

Green technology is becoming a driving force in the chemical industry because of the current need to decrease pollution caused by the petrochemical process and the

future need to replace the hydrocarbon-based economy with a renewable, environmentally sound, bio-based economy.

Succinic acid, being an intermediate of the tricarboxylic acid (TCA) cycle and one of the end products of anaerobic metabolism, is synthesized in almost all microorganism, plants, and animal. Bio-based succinate production also involves CO_2 fixation, because the synthesis of 1 mol of succinic acid requires the fixation of 1 mol of CO_2. Thus, in addition to being based on renewable resources, succinate production has the environmental benefit of using a greenhouse gas as a substrate.

Table 1.2 presents data regarding fermentation efficiency and operating conditions reported for various succinic acid producing strains by conversion of glucose.

***Table 1.2**: Production data of succinic acid by microorganisms. Carbon source: glucose*

Microorganism	**Mode of operation**	**SA**			**Reference**
		Concentration g/L	Yield g/g	Productivity g/L*h	
Anaerobiospirillum succiniproducens	Anaerobic batch	32.2	0.99	1.2	Nghiem et al., 1997
Actinobacillus succinogenes	Anaerobic batch	80	1.70	0.9	Guettler, 1996
Corynebacterium glutammicum	Anaerobic batch	23	0.19	3.8	Okino et al., 2005
Mannheimia succiniproducens	Anaerobic batch	13.5	0.68	1.8	Lee et al., 2002
Escherichia coli*	Anaerobic batch	71.5	1.00	0.9	Jantama et al., 2008
Basfia succiniproducens	Anaerobic batch	5.8	0.60	1.5	Scholten and Dägele, 2008
Saccharomyces cerevisiae*	Aerobic batch	3.5	0.07	0.2	Raab et al., 2010
Yarrovia lipolitica **	Aerobic batch	45.5	0.36	0.3	Yuzbashev et al., 2010

*Genetically modified strain
**Carbon source is glycerol

The first approach for microbial production of succinic acid was the engineering of the mixed acid fermentation of *Escherichia coli* (Chatterjee et al., 2001). Fermentative succinic acid production has been accomplished by both wild-type and genetically engineered strains. *S. cerevisiae* is a well characterized eukaryotic microorganism that has been most widely used for industrial bioethanol production. Moreover, its genome is sequenced, it is physiologically well characterized, and can produce organic acids even at the low pH that facilitates downstream. Many tools for genetic improvement are established. These features *make S. cerevisiae* suitable for the biotechnological production of succinate. Due to this fact, attempts to engineer *S. cerevisiae* for the succinate production were made. Only few Gram-positive bacteria like *Corynebacterium glutamicum* and *Enterococcus faecalis* have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by

disruption and replacement of genes, and their optimal culture conditions were developed.

Actinobacillus succinogenes, Basfia succiniciproducens and Mannheimia succiniciproducens are the most promising wild-type bacterial strains because they: i) are able to metabolise numerous carbon sources; ii) are facultative anaerobes; iii) can achieve high fermentation efficiency; and iv) are classified as biosafety level 1 microorganisms by DSMZ and ATCC. These strains have been isolated from the rumen.

1.3.1 Bio-based production of SA: commercial status

Nowadays, there are four producers of bio-based succinic acid - Myriant, BioAmber, Succinity and Reverdia (see Figure 1.3) - which have formed joined ventures with several other commercial entities.

Myriant is employing an engineered *E. coli* strain for the production of SA and it has been operating a 15 ktonnes capacity plant since 2013.

BioAmber has initially selected *E. coli* as producing organism, but more recently it appears to have switched to *Candida krusei* as current SA producer. It has been operating a 30 ktonnes capacity plant since 2014.

Succinity (joint venture between BASF & Corbion-Purac) is employing the engineered *B. succiniciproducens* strain for the production of SA. It has been operating a 10 ktonnes capacity plant since 2013. This wild-type strain – characterized by high yield of 0.75 mol of SA per mol of glucose - was further optimized through metabolic flux analysis and subsequent metabolic engineering (Becker et al., 2013).

Reverdia (joint venture Roquette and DSM) has started production in a commercial plant of about 10 ktonnes per year in December 2012. They use a proprietary *S. cerevisiae* strain and low pH fermentation technology.

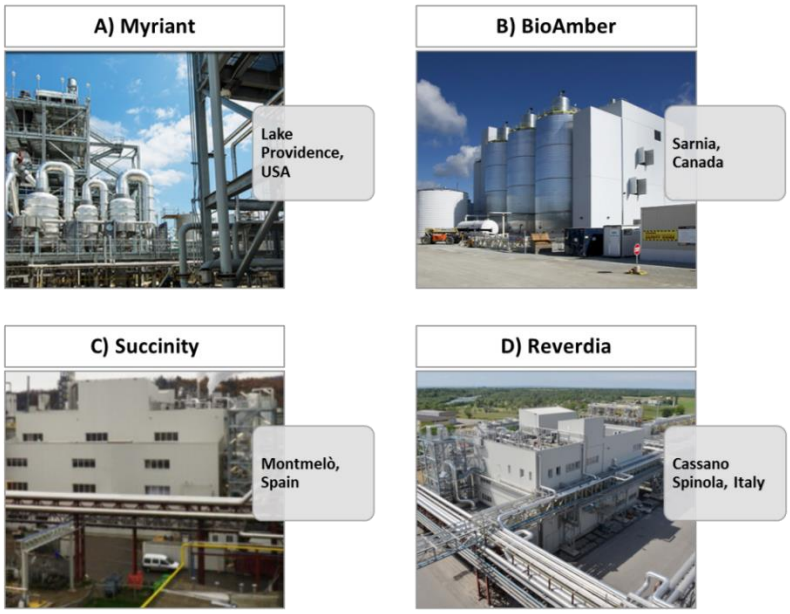

***Figure 1.3**: Bio-based succinic acid production plants: A) Myriant in USA; B) BioAmber in Canada; C) Succinity in Spain and D) Reverdia in Italy.*

1.4 *Actinobacillus succinogenes* as SA producer

Actinobacillus succinogenes is a Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod, which was isolated from bovine rumen (Guettler et al., 1999) and taxonomically was placed in the Pasteurellaceae family, based on 16S rRNA amplification. It was first isolated from a Michigan State University cow. *A. succinogenes* is mesophilic and grows well at 37–39°C in chemically defined media. It is able to utilize a wide range of C5 and C6 sugars (glucose, xylose, arabinose, mannose, galactose, fructose) as well as various disaccharides and other carbon sources (sucrose, lactose, cellobiose, mannitol, maltose and glycerol). Moreover, is a moderate osmophile and has good tolerance to a high concentration of glucose, which is beneficial for fermentation.

A. succinogenes, is a promising biocatalyst for industrial succinate because it produces succinate at the highest concentration reported in the literature. However, succinc acid is produced as part of a mixed acid fermentation: formate and acetate at high concentration are produced as well.

Figure 1.4 shows the metabolic pathway leading to the production of succinic acid in *A. succinogenes*. Phosphoenolpyruvate (PEP) is one of the central intermediates during this mixed acid fermentation. It is either converted into pyruvate, resulting in the formation of the fermentation products acetate, formate, ethanol, and lactate (C3 pathway), or it is converted into oxaloacetate (OAA), resulting in the formation of succinate (C4 pathway), with malic enzyme and oxaloacetate (OAA) decarboxylase forming reversible shunts between these pathways.

The oxidative pentose phosphate pathway (OPPP) has limited contribution in the catabolism of glucose.

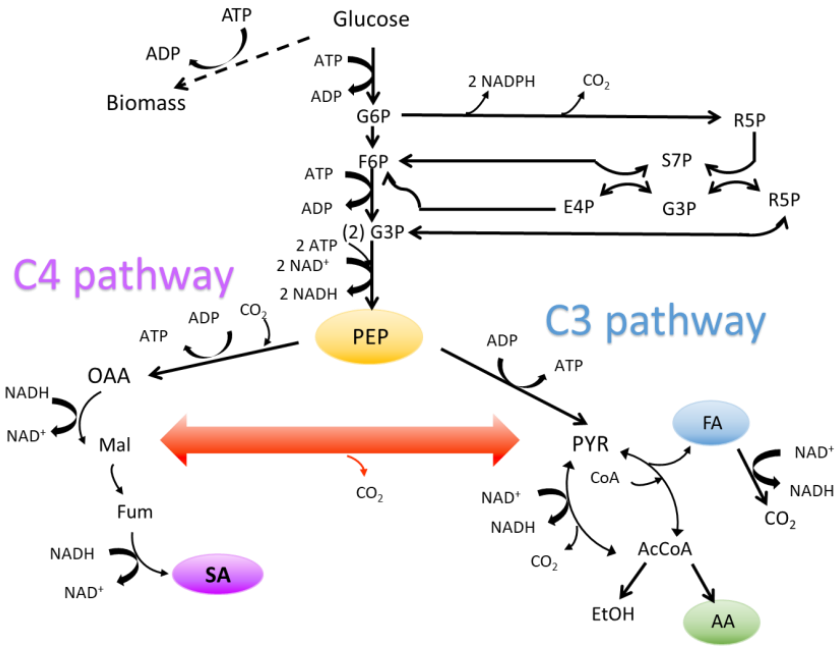

***Figure 1.4**: Metabolic map of the central metabolism of A. succinogenes.*

Theoretically, 1 mol of succinic acid can be produced from 0.5 mol of glucose, 0.6 mol of xylose or 1 mol of glycerol and the fixation of 1 mol of CO_2. However, 0.5 mol of glucose can only synthesize 1 mol of NADH, and the generation of 1 mol of succinic acid requires 2 mol of NADH. This means that the additional reducing

capacity (i.e. NADH) required in the C4 pathway should be supplied by other parts of the metabolism (e.g. glycolysis, C3 pathway).

Bradfield and Nicol, (2014)suggested that the maximum glucose to succinic acid conversion yield can be calculated from the following stoichiometric Equation (1):

$$C_6H_{12}O_6 + \frac{6}{7}CO_2 + NADH \rightarrow \frac{12}{7}C_4H_6O_4 + NAD^+ + ATP + \frac{6}{7}H_2O \quad (1)$$

If expressed in mass units, the above equation results in a maximum theoretical succinic acid yield of 1.12 g succinic acid per gram glucose, if no biomass or by-products are formed and considering the effect of reduction potential.

1.5 Bioprocessing aspects of SA production by *A. succinogenes*

1.5.1 Renewable resource for SA

Various industrial waste and by-product streams (e.g. sugarcane molasses, cheese whey, crude glycerol from biodiesel production, wheat milling by-products, sake lees) and agricultural residues (e.g. corn fiber and corncob, sugarcane bagasse, bio-waste cotton) have been evaluated for the production of succinic acid. *A. succinogenes* also requires significant amount of complex and expensive nitrogen source (i. e. yeast extract). To reduce the cost of the fermentation feedstock, cheap nitrogen source need be used, such as corn steep liquor (CSL) or nitrogen-rich renewable resource that also provides the carbon source (e.g. wheat milling by-products, waste bread). The utilization of agro-food wastes (AFWs) and by-product streams may also supply other nutrients, such as minerals and vitamins.

The fermentation substrates can be divided into two main categories: non lignocellulosic substrates and substrates derived from lignocellulose or the lignocellulose itself.

Non lignocellulosic substrates

Aquatic biomass is considered as an ideal feedstock for biorefineries in particular macroalgae. The production of these biomass does not requires the use of arable land and fertilizers as it happens for energy crops or lignocellulosic biomasses. Therefore, aquatic biomass production minimize the competition for land, food and feed production. Another advantage of aquatic biomass is that different macroalgae species (e.g. *Laminaria digitata* and *Saccharina latissima*) can reach carbohydrate content up to 60% DM which makes them suitable substrates for the production of building block chemicals and bioenergy through a biorefinery approach (Efremenko et al., 2012).

Alvarado-Morales et al. (2015) reported succinic acid production from *Laminaria digitata* hydrolysate by *A. succinogenes* 130Z. They obtained a yield of 86.5% (g_{SA}/ g of total sugars) and an overall productivity of 0.50 g/L* h.

Glycerol has also appeared as a promising feedstock for succinic acid production as it is a cheap and highly abundant byproduct of the biodiesel industry. Despite its strengths, the cost of raw glycerol has shot up in the past two years, making it less competitive as compared to crop straw wastes. However, glycerol can be used in biosuccinic acid production without cost-consuming pretreatment procedures that are needed for lignocellulosic wastes. Some investigation on succinic acid production using glycerol as carbon source by means of different microorganism are reported in literature (Carvalho et al., 2014; Gao et al., 2016; Scholten et al., 2009).

Waste and by-product streams from the food-industry could be employed for the production of succinic acid. Annual global food waste is estimated to be about 1.3 billion t, about one third of the total food production for human consumption (www.fao.org, 2014).

High sugar content beverages (HSCBs) such as fruit juice and soft drinks can be used are an example. They contain sugars such as sucrose, fructose and glucose. The European market size for HSBCs is about 70 Mt per year (Www.zenithinternational.com) and about 0.1% of the production – e.g., beverages outside commercial targets, process wastewaters, seizures of badly repaired or illegal goods, and stocks after their expiration date – may request remediation.

Lignocellulosic substrates

Lignocellulosic wastes are quite abundant and they are obtained from agricultural and industrial resources. A large amount of research is dedicated to biofuels and biochemicals production from lignocellulosic biomass.

The raw material are considered a good substrate for the production of organic acids, if they have the following characteristics: available abundantly throughout the year, renewable, cheap, produces less amounts or no by-product formation, high production rate, low level of contaminants and no competing food value (Procentese et al., 2016).

Lignocellulosic biomass consists of cellulose, hemicellulose and lignin which form a complex structure. Pretreatment and hydrolysis of lignocellulosic biomass are required to release the fermentable hexoses and pentoses sugars and they should be carried out via combined thermo-chemical and enzymatic treatments. However, few microorganisms can metabolize the pentoses derived from these raw materials.

Many investigation are present in literature regarding succinic acid production from cheap and renewable lignocellulosic biomass, which have been reviewed by Akhtar et al. (2014).

Table 1.3 reports an overview of the most relevant investigations regarding succinic acid production from renewable feedstocks.

The production of succinic acid via both separate enzymatic hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) has the potential to reduce the use of non-sustainable resources and carbon footprint. SHF has been widely used in the production of succinic acid from lignocellulosic materials such as corncobs, corn stover, rice straw, wheat straw, bagasse, and empty fruit bunches. Conversely, data on SSF using lignocellulosic biomass for succinic acid are rather limited.

***Table 1.3**: Bio-based succinic acid production using different raw materials and microbial strain*

Substrate	Microorganism	Operation mode	SA			Reference
			Concentration (g/L)	Productivity (g/L*h)	Yield (g_{SA}/g_{sub})	
Corn fiber hydrolizate	*A. succinogenes*	Anaerobic batch	70.3	0.63	0.96	Chen et al., 2011
Corn stalk and cotton stalk	*A. succinogenes*	Anaerobic batch	15.8	0.60	1.23	Li et al., 2010
Corn stover	*A. succinogenes*	Anaerobic batch	56.4	n.a.	0.73	Li et al., 2011
	B. succiniproducens	Anaerobic batch	30.0	0.43	0.69	Salvachúa et al., 2016
Sugarcane bagasse hydrolyzate	*A. succinogenes*	Anaerobic batch	22.5	1.01	0.43	Borges and Pereira, 2011
Corn staw	*A. succinogenes*	Anaerobic fed-batch	53.2	1.21	0.83	Zheng et al., 2009
Cane molasses	*A. succinogenes*	Anaerobic fed- batch	55.2	1.15	0.81	Liu et al., 2008
Whey	*A. succiniproducens*	Anaerobic fed-batch	34.7	1.02	0.91	Samuelov et al., 1999
	M. succiniproducens	Anaerobic continuous	6.4	3.9	0.69	Lee et al., 2003
	A. succinogenes	Anaerobic batch	21.3	0.43	0.44	Wan et al., 2008
Wood hydrolizate	*M. succiniproducens*	Anaerobic batch	11.7	1.17	0.56	Kim et al., 2004
Orange peel	*F. succinogenes*		1.75	2.02	0.04	Li et al., 2010
Microalgal hydrolizate	*A. succinogenes*	Anaerobic batch	33.0	1.27	0.75	Alvarado-Morales et al., 2015
	A. succinogenes	Anaerobic batch	36.8	3.9	0.92	Marinho et al., 2016
	E. coli	Dual- phase fermentation	17.4			Bai et al., 2015
Crude glycerol	*B. succiniproducens*	Anaerobic continuous	5.21	0.09	1.02	Scholten et al., 2009
	Y. lipolytica	Aerobic fed- batch	160.2	0.40		Gao et al., 2016
Carob pods	*A. succinogenes*	Anaerobic fed-batch	19.0	1.43	0.94	Carvalho et al., 2016

1.5.2 Succinc acid production kinetics

Succinic acid production by fermentation is affected by substrate and product inhibition. Inhibition on growth by metabolic product formation could be a result of two mechanisms: (1) regulation of homeostasis and (2) membrane breakdown due to osmotic stress. The lipophile, undissociated form of weak acids can enter the cell at neutral pH values. The concentration of undissociated form of each acid in the fermentation broth is determined by several parameters, such as the pKa values, the concentrations of the produced acids, the broth pH, and temperature of the medium. pH homeostasis is maintained through the synthesis of excessive ATP and the regulation of H+-ATPase, which expels protons from the cytoplasm (proton motive force) against the concentration gradient. High osmotic stress occurs when the medium contains high concentrations of sugars and/or minerals, causing cell shrinkage, subsequent breakdown of the membrane and cell death.

The presence of more than one organic acid can cause synergistic effects which might result in more severe inhibition phenomena.

The main investigations on the kinetic of succinic acid production are reported hereinafter. Lin et al. (2008) proposed a growth kinetic model for *A. succinigenes* that includes both substrate and products inhibition. The model describes the inhibitory kinetics caused by the produced acids during batch fermentation. Pateraki et al., (2016) presented unstructured models - including substrate and product inhibition – that predict the growth and succinic acid production profiles of *A. succinogene*s and *Basfia succiniciproducens* on a mixture of C5 and C6 sugars, miming the composition of a spent sulphite liquor. Song et al., (2008) proposed a kinetic model for the batch production of succinic acid from glucose by *M. succiniciproducens* MBEL55E. All these studies used a modified Monod model, including terms for both substrate and product inhibitions.

As regards the substrate inhibition, biofilm and suspended cultures may be characterized by a different behaviors. Established biofilms appear to be unaffected by the initial glucose concentration (Yan et al., 2013) while the lag time of the suspended cultures (no lag for biofilm cultures) is clearly influenced by the initial glucose concentrations (Lin et al., 2008a).

1.5.3 Bioreactor type and mode of operation

Many different fermentation strategies for the production of succinic acid have been described in recent literature, including utilization of immobilized biocatalysts, integrated fermentation and separation systems and batch, fed-batch and continuous modes of operation.

Table 1.4 reports an overview of different bioreactor configuration used in literature for SA production, the carbon source and the process performances.

Mostly batch cultures are employed as a production system, although higher concentration and productivity are obtained by fed-batch and continuous culture systems. The highest volumetric production rate, 10.4 g/L*h was obtained in a continuous culture of *A. succiniciproducens* with integrated membrane for cell recycling at a dilution rate of $0.98h^{-1}$ (Meynial-salles et al., 2008). Titers up to 146 g/L were obtained in a cell recycling fed batch culture of *C. glutamicum* (Okino et al., 2008). In an *E. coli* fedbatch fermentation the highest described yield of 1.1 g/g substrate (Vemuri et al., 2015) was obtained. None of the described production systems are homo-fermentative succinate production systems.

During the past few years, continuous succinate fermentation has been carried out in literature, but only the natural producers such as *M. succiniciproducens* (Kim et al., 2004), *A. succiniciproducens* (Lee et al., 2010), and *A. succinogenes* (van Heerden and Nicol, 2013) have been tested.

***Table 1.4**: Summary of different reactor configuration and operation mode used in literature for SA production*

Bioreactor	Operation mode	Microorganism	Carbon source	SA			Reference
				Concentration g/L	Yield g/g	Productivity g/L*h	
Sealed tubes	Batch	*A.succinogenes*	Glucose	4.2	0.46	0.3	Mckinlay et al., 2005
5 L stirred bioreactor	Fed-batch	*A.succinogenes*	Glucose	60.2	0.75	1.3	Liu et al., 2008
Cylindrical bioreactor with external recycle	Continuous	*A.succinogenes*	Glucose	48.5	0.91	-	Bradfield and Nicol, 2014
Biofilm reactor with plastic composite support	Batch and repeated-batch	*A.succinogenes*	Glucose	35.1	0.68	0.7	Urbance et al., 2004
Fibrous-bed bioreactor	Fed-batch	*A.succinogenes*	Glucose	98.7	0.89	2.8	Yan et al., 2013
1.8 L stirred bioreactor	Batch	*A.succinogenes*	wheat flour hydrolizate	16.0	0.19		Du et al., 2007
2.5 L stirred bioreactors	Batch	*A.succinogenes*	Cheese whey	21.0	0.57	0.4	Wan et al., 2008
External membrane cell recycle reactor	Continuous	*M. succiniproducens*	Glucose	12.8	0.64	1.3	Kim et al., 2009
2.5 L stirred bioreactor.	Continuous	*M. succiniproducens*	Wood hydrolysate	7.0	0.55	3.2	(Kim et al., 2004)

1.6 Biofilm reactors for the production of chemicals and fuels

Biomass concentration is a rate-limiting issue in anaerobic fermentations. Due to the slow growth rate of the anaerobic microorganisms, production rate and productivity of cell/fermentation-products are typically extremely low. Thus, to increase productivity, the biomass concentration in the bioreactor must be increased.

Immobilized cell bioreactors are designed to retain high biomass concentration in the reactor volume. Cells may be immobilized by three techniques: entrapment, covalent bond formation and adsorption. Entrapment and covalent bond formation require use of chemicals that may restrict propagation or increase cell concentration in the reactor and it may increase the process investments. The third technique – a natural process – consists in the adhesion of cells to supports and growth of firm biofilm (Corona-González et al., 2014; Qureshi et al., 2000). The latter technique is widely adopted in processes characterized by huge throughput of inexpensive products (Gross et al., 2007; Qureshi et al., 2004b).

In nature, biofilms exist primarily as complex multi-species communities of bacteria in which each species fills an ecological niche within the biofilm depending on its metabolism and morphology (Stoodley et al., 2002). Multi-species biofilms are used industrially to achieve several aims, including the treatment of wastewaters to remove organics and heavy metals (Kumar et al., 2008; Russo et al., 2010). Single specie biofilms are used industrially to produce chemicals (Qureshi et al., 2005).

1.6.1 Biofilm formation

A biofilm is defined as an aggregate of microorganisms embedded in an organic polymer of microbial origin and attached to a solid surfaces (Donlan, 2002). Generally, the formation of a stable biofilm follows four main stages: initial attachment, irreversible attachment by the production of extracellular polymeric substances (EPS), early development, and maturation of biofilm architecture (see Figure 1.5) (Stoodley et al., 2002).

The first stage of biofilm growth is strongly influenced by interaction of the cell wall with the solid surface that, in turn, is related to flow conditions in the neighbouring liquid phase (stagnant, laminar flow, turbulent flow), with the surface properties (roughness and charge) and with the phenotype expressed by the planktonic cells. The cells suspended in the liquid are transported to the surface by diffusion, convection, or self- motility, and form weak reversible adhesion with the solid surface. After initial association with the surface, a planktonic bacterial cell can dissociate from the surface and resume the planktonic state or become irreversibly attached to the surface. Irreversible attachment involves the production of exopolysaccharides (EPS), that bind the cell to the surface and protect it from the surrounding environment.

The third stage of biofilm formation, growth, is highly affected by the factors including nutrient availability and diffusion, shear force of bulk flow, and growth rate of microorganisms.

Phenomena like erosion or sloughing can occur during biofilm growth. Erosion is defined as the removal of small portions of biofilm and is caused by liquid shear forces. Sloughing, in contrast, is the random detachment of large portions of the biofilm as a result of rapid change or depletion of nutrients.

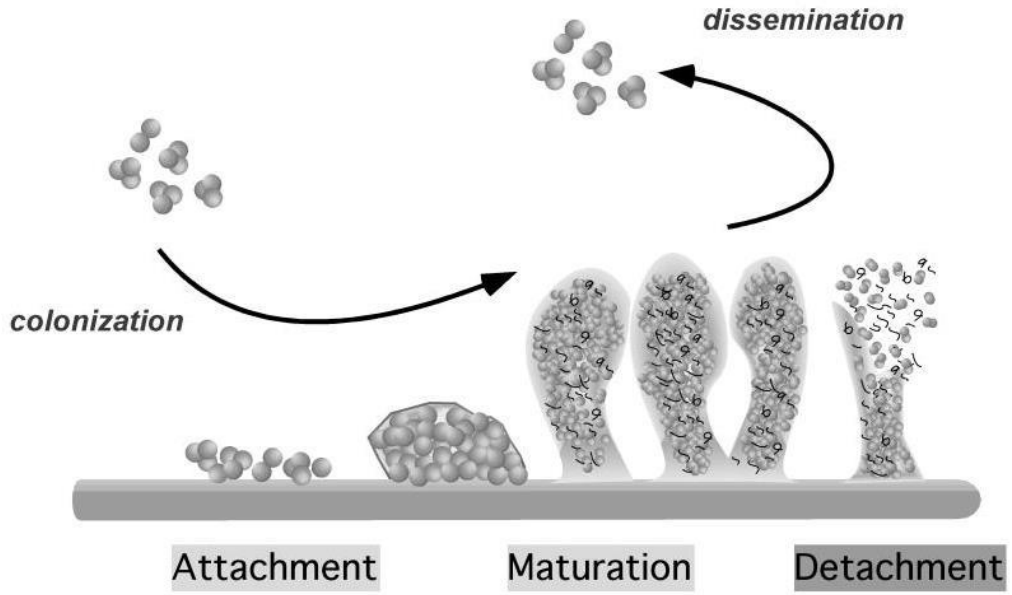

Figure 1.5*: Sketch of biofilm formation on a flat surface under stagnant conditions. Available on-line on www.en.infektiologie.usz.ch*

The structure of a biofilm is determined by the combination of several factors: nutrients availability and diffusion, attraction force between surface and microorganisms, EPS production and intercellular adhesion, microorganisms growth, shear force, temperature, and pH (Horn et al., 2003; Picioreanu et al., 1998)

1.6.2 Types of biofilm reactors

Biofilms are used in various types of reactors such as continuous stirred tank reactors (CSTRs), packed bed biofilm reactors (PBBRs), fluidized bed biofilm reactors (FBBRs), airlift reactors (ALRs), upflow anaerobic sludge blanket (UASB) reactors, and expanded granular sludge bed (EGSB) reactors, etc. (Raganati et al., 2013; Viggiani et al., 2006). The potential of using microbial aggregates/biofilms as an effective tools to enhance productivity in bioprocesses has been demonstrated (e.g. in the food industry) and it has stimulated additional research effort aimed at expanding their use in bioreactor technology.

CSTR are stirred using a mechanical device such as impeller. CSTRs cannot be packed with the adsorbent support covered by biofilms , but they can be used with a fibrous bed support.

PBBRs are packed bed of selected support material. The biofilm formation may take from a few to several days depending on the strain, substrates, and support. Provided the formation of the biofilm, the reactor is operated under continuous conditions. Reactors are usually fed at the bottom, getting products at the top. These reactors may be easily clogged as a consequence of the excessive cell growth. Indeed, TBRs are feed from the top of the reactor.

In FRBs particles move up and down within the expanded bed in the well defined zone of the reactor and cell growth occurs around the adsorbent particles.

Airlift reactors contain two concentric tubes, a riser (an inner tube) and a downcomer (an outer tube). In these reactors, mixing is achieved by circulating essentially air at the bottom of the reactor.

Upflow anaerobic sludge blanket (UASB) reactors (contain granular biofilm particles) are used for anaerobic treatment of wastewater/industrial effluents.

A. succinogenes is well known to self-adhere to support surfaces and form biofilms under prolonged operation. All continuous studies on *A. succinogenes* resulted in unavoidable biofilm formation (Bradfield and Nicol, 2014; Urbance et al., 2004; van Heerden and Nicol, 2013). Accordingly, adsorption can be used as cheap self-immobilization technique for this SA producing microorganism.

1.6.3 Applications of biofilm reactors

Biofilm reactors have been utilized for many purposes, including wastewater treatment, animal and plant cell cultures, bioremediation and the production of fuels and chemicals.

Many examples of biofilm reactors that have been successfully employed for the production of ethanol are available in literature. Mainly, adsorption was used as immobilization technique with both a bacterial species, *Zymomonas mobilis*, and the well-known yeast *Saccharomyces cerevisiae*.

In a work by Tyagi and Ghose, (1982) cells of *S. cerevisiae* adsorbed into a natural support were used to produce ethanol from molasses in a packed bed continuous bioreactor. In this biofilm reactor, the productivity was about 10-fold higher compared to the free cell reactor system. A genetically engineered *Escherichia coli* strain was also employed in a biofilm reactor to produce ethanol from xylose, obtaining improved productivity than batch and free cell continuous reactors (Qureshi et al., 2004a).

Production of butanol in biofilm reactors has been extensively investigated. The biofilm reactor systems that have been used for butanol production include vertical packed bed reactor (PBR), horizontal PBR, compartmentalized reactor, double series reactors, and

FBR. The most investigated microorganism for this application are *Clostridium acetobutylicum* and *Clostridium beijerinckii*. The supports utilized include bonechar, glass beads, glass wool, polypropylene tow, stainless steel wire balls, clay brick.

In a recent publication by Raganati et al. (2016), butanol was produced in a series of four packed bed biofilm reactors, using lactose as carbon source. The first reactor of the series (fed with fresh medium) was kept under acidogenesis conditions and the three others were kept under solventogenesis conditions. With this system, butanol productivity and concentration values were the highest ever reported in literature.

Biofilm reactors have also been investigated to improve the productivity in 2,3- butanediol fermentation, with *Klebsiella pneumoniae* as biocatalysts.

Biofilm reactors have been applied for the production of organic acids. Some examples includes acetic acid, citric acid, succinic acid, lactic acid, fumaric acid. Cotton et al., (2001) used *Lactobacillus casei* subsp. *rhamnosus* to continuously produce lactic acid in a immobilized-cells biofilm reactor with a unique plastic composite support (PCS) that stimulates biofilm formation.

Few studies available in literature investigated the possibility to use biofilm reactors for polysaccharide production, such as cellulose, pullulan and xanthan.

2. AIM OF THE THESIS

The study carried out during the present Ph.D. program aimed at investigating succinic acid production process by fermentation. The work was carried out at the Dipartimento di Ingegneria Chimica, dei Materiale e della Produzione Industriale of the Università degli Studi di Napoli 'Federico II'. The activities were articulated along two paths: i) the characterization of the succinic acid fermentation process as regards the concentration and yield using renewable feedstock and waste-sugar streams; ii) the development of a continuous biofilm reactor for the intensive succinic acid production. A commercial strain was investigated. *Actinobacillus succinogenes* DSM 792 was selected for its ability to produce succinic acid at high concentration and yield from a broad variety of carbon source.

Characterization of the succinic acid fermentation process as regards the concentration and yields

The study was aimed at the assessment of both the fermentation performance - in terms of succinic acid concentration and yield – with reference to two classes of feedstocks: 1) sugars typically present in lignocellulosic hydrolysates (glucose, mannose, arabinose and xylose); 2) high sugar content beverages (HSCBs).

As regard the lignocellulosic derived sugars, tests were carried out at different sugar concentration and the conversion process was characterized in terms of metabolite production (succinic acid, formic acid and acetic acid), sugar conversion, pH and cell concentration. Test were also carried out with sugar mixtures (GMAX) that mime the composition of lignocellulosic hydrolysate to assess the possible synergic/interfering effects of sugars and other hydrolyses products.

The investigation was carried out with batch of free *A. succinogenes* cells. Batch tests were focused on the preliminary characterization of the fermentation with the aim to highlight the relevant features of the process.

Development of a continuous process for the succinic acid production

Continuous fermentation tests carried out in a CSTR were aimed to the systematic characterization of the kinetics of cell growth and succinic acid production. The continuous fermentation were carried out by feeding the CSTR with a glucose bearing medium at a wide interval of dilution rate.

Continuous cultures were carried out under a wide interval of operating conditions to characterize the fermentation process in terms of cells, metabolites and glucose concentrations. The study was aimed at the assessment of a kinetic model and yields for cell growth and metabolites.

The activity aimed at the intensive succinic acid production regarded the design, set-up, operation and optimisation of a packed bed biofilm reactor (PBBR). The effects of the operating conditions - dilution rate (D) and medium compositions (glucose, GX, xylose, GAX media) - on the PBBR performances were investigated. The effect of HMF and furfural, the main inhibitors found in lignocellulosic hydrolysate, on succinic acid production was also investigated.

Production of bio-based adipic acid at Chalmers University of Technology

This research activity was focused on the development of a microbial cell factory for adipic acid production. The objectives were i) *in vitro* characterization of two fumarate reductase as potential enzymes to catalyse one of the enzymatic steps in the biosynthetic pathway to produce adipic acid; ii) the assessment of the physiology and robustness of different yeast strains in presence of adipic acid to evaluate their tolerance level toward the acid. The activity described above were carried out at Department of Biology and Biological Engineering of Chalmers University of Technology. They are reported in details in the *Appendix* section.

3 SA PRODUCTION FROM RENEWABLE FEEDSTOCKS

The activity focused on the characterization of the succinic acid fermentation process using two class of feedstocks: 1) sugars typically present in lignocellulosic hydrolysates (glucose, mannose, arabinose, and xylose); 2) high sugar content beverages (HSCBs). The section 3.1 reports the results of preliminary tests carried out with *A. succinogenes* and the sugars typically present in a lignocellulosic hydrolysate: glucose, mannose, arabinose, and xylose. The tests were carried out with solutions of single sugar at two concentrations. The section 3.2 reports the results of a systematic characterization of succinic acid fermentation process with solution of single sugars and sugar mixtures to mime lignocellulosic hydrolysate composition (glucose, mannose, arabinose and xylose-GMAX). The section 3.3 reports the results of tests carried out with high sugar content beverages (HSCBs) as carbon source to produce succinic acid. The beverages tested were fruit juices, soft drinks and syrups.

3.1 Succinic Acid Production from Hexoses and Pentoses by Fermentation of *Actinobacillus succinogenes*

Mariateresa Ferone, Francesca Raganati, Giuseppe Olivieri, Piero Salatino, Antonio Marzocchella*

Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale – Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

Abstract

Succinic acid is a precursor for many chemicals in the food, pharmaceutical, cosmetic, and biodegradable plastics industries. Nowadays, the biotechnological route to produce succinic acid is focused on processes based on the fermentation of natural materials characterized by high fraction of carbohydrates. *Actinobacillus succinogenes* is proposed in the literature as a promising strain for the production of succinic acid. Indeed, *A. succinogenes* may utilize a wide spectrum of carbohydrates as carbon sources. Moreover, the CO_2 required for the fermentation allows to include the biotechnological route among the carbon capture and conversion processes.

A key issue of fermentation processes aimed at producing succinic acid is the feedstock cost. Pre-requisites of the feedstock are: availability at high mass flow rate over all the year, and non-competitive with (human and animal) food. Lignocellulosic biomass is a potential feedstock because it fulfils the listed pre-requisites.

This contribution reports the characterization of succinic acid fermentation by *A. succinogenes* 130Z adopting as carbon source sugars representative of the hydrolysis of lignocellulosic biomass: glucose, mannose, arabinose, and xylose. Batch fermentation tests were carried out using single sugar as carbon source for a systematic characterization of the conversion process of the investigated sugars.

The conversion process was characterized in terms of concentration of biomass, sugars, and acids as well as of pH. The time-resolved data were processed to assess the sugar conversion, the succinic acid yield, and the productivity for each of the investigated sugars.

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A. succinogenes was able to convert all investigated sugars into succinic acid using $MgCO_3$ as an indirect CO_2 source. Glucose was the sugar characterized by the best performance when the initial concentration was set at 40 g/L. The performances did not depended on the investigated sugars if the initial concentration was quite low (5 g/L).

Introduction

The limited nature of fossil reserves, the increasing worldwide demand for durable consumer goods, and the increasing environmental concerns are driving forces for a net reduction of the worldwide consumption of resources. A contribution to mitigate the catastrophic effects of the listed issues may be offered by efficient biotechnological processes converting renewable resources (Willke and Vorlop, 2004).

Succinic acid (SA) - a dicarboxylic acid also known as amber acid, is a well-established bio-based platform for chemicals and intermediates (Bozell and Petersen, 2010). The global market of succinic acid has been estimated to range between 30'000 and 50'000 tonnes per year in 2011. Moreover, the market is expected to grow at an annual rate of 18.7 per cent from 2011 to 2016 (www.marketsandmarkets.com, 2012). Main markets of the succinic acid are: i) surfactants, additives, foaming agent and detergent. This is the largest market; ii) the use as an ion chelator to prevent corrosion and pitting in the metal industry; iii) the food market where it is used as acidulate agent, pH regulator, anti-microbial and flavouring agent; iv) the pharmaceutical industry where it is used as an additive for the production of vitamins, antibiotics and amino acids (Zeikus et al, 1999). Moreover, the terminal carboxylic acid groups open up numerous possibilities for further processing. Major developments include polymerisation of SA with its hydrogenated diol product (1,4-butanediol) to produce the biodegradable plastic polybutylene succinate (PBS).

The conventional process to produce succinic acid is via the (petrol)chemical route from butane or benzene via the conversion of maleic anhydride to succinic anhydride and successive hydrolysis. It may also be produced by the oxidation of 1,4-butanediol and by the carbonylation of ethylene glycol. Recently, the trend is the replacement of the conventional (petrol)chemical route with the biotechnological route to produce succinic acid by microbial fermentations. The biotechnological route may replace the petrochemical-based route with marked environmental benefits. Indeed, the production of succinic acid may adopt renewable resources (e.g. biomass and CO_2 streams) as feedstocks. Therefore, the succinic acid production via the biotechnological route contributes to reduce the request of fossil resources and mitigate the release of CO_2 in the atmosphere (McKinlay et al., 2005).

Several succinic acid producer gram-negative bacteria have been isolated in anaerobic environments. The most documented SA producers are wild type strains of *Actinobacillus succinogenes* (Guettler et al., 1999)*, Mannheimia succiniciproducens* (Lee et al., 2002)*, Anaerobiospirillum succiniciproducens* (Samuelov et al., 1991) and various recombinant strains of *Escherichia coli* (Jantama et al., 2008) and *Corynebacterium glutamicum* (Litsanov et al., 2012). *A. succinogenes* - a Gram-negative capnophilic bacterium isolated from the bovine rumen - is considered to be one of the most promising strains for industrial succinic acid production because of its ability to produce a comparatively large amount of succinic acid. Moreover*, A. succinogenes* can utilize a

variety of carbohydrates as carbon sources, including glucose, lactose, xylose, arabinose, cellobiose, and other sugars (Guettler et al., 1999; Van der Werf et al., 1997). To the author knowledge, no systematic investigation has been carried out to characterize the fermentation of these sugars.

A key parameter for the success of the bioprocess on industrial scale is the cost of the fermentation substrate. Potential feedstocks for the production of succinic acid by fermentation are lignocellulosic materials as wood and agricultural residues (Akhtar et al., 2014). Lignocellulose is the most abundant renewable resource on the planet and it has great potential as substrate for fermentation because of the un-competitiveness with food resources. It use as feedstock requires a pretreatment and hydrolysis to release the simple sugars to be fermented: glucose, mannose, xylose, and arabinose.

This contribution reports a recent activity on succinic acid production by fermentation of glucose, mannose, xylose, and arabinose. This activity is part of the national research project “Development of green technologies for production of BIOchemicals and their use in preparation and industrial application of POLImeric materials from agricultural biomasses cultivated in a sustainable way in Campania Region – BIOPOLIS” PON03PE_00107_1/1. The preliminary characterization of the fermentation process was carried out in batch tests. Each test was characterized in terms of succinic acid yield and maximum concentration.

Materials and Methods

Microorganism and media

Actinobacillus succinogenes DSM 22257 by DSMZ was used. Stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C.

The thawed cells were inoculated into 10 mL Brain Heart Infusion Broth medium in 15 mL Hungate tubes (pre-cultures). Vials were inoculated by a syringe to ensure anaerobic conditions. The cultures were carried out for 24h under agitation (rotary shaker at 150 rpm) at 37 °C .

The composition of the fermentation medium was: 5 g/L yeast extract, 1 g/L NaCl, 0.3 g/L Na_2HPO_4, 1.4 g/L NaH_2PO_4, 1.5 g/L K_2HPO_4, 0.2 g/L $MgCl_2•6H_2O$. 0.23 g/L $CaCl_2•2H_2O$ (Van Heerden and Nicol, 2013). $MgCO_3$ was also supplied in the fermentation medium at concentration of 5 or 20 g/L to act as an indirect CO_2 source and to buffer the pH during growth. The medium was heat sterilized (15 min at 121 °C) in anaerobic bottles sealed with a butyl rubber stopper with a nitrogen headspace (Raganati et al., 2014). $MgCl_2$, $CaCl_2$ and sugars were prepared separately as concentrated stocks ($MgCl_2$ and $CaCl_2$ at 100 time the operating concentration; sugar at 300g/L), sterilized by filtration and supplemented aseptically to the autoclaved medium.

Batch fermentation tests

The experiments were carried out in 100 mL anaerobic bottles containing 75 mL of medium. The medium was inoculated with a 6.25 % (v/v) suspension of actively growing pre-cultures. The bottles were placed on a rotary shaker at 150 rpm and incubated at 37°C. Fermentation samples were taken every 2-3 h to measure optical density (OD), metabolites concentration and pH. The sugars investigated were: glucose, mannose,

arabinose, and xylose. Each test was carried out in duplicate and the mean values are reported as results.

The batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and metabolites (acids and solvents) production. In particular, the measured data were worked out to assess the following parameter:

- overall sugar conversion (ξ_S), the ratio between the sugar converted and the initial sugar $(S_0 - S)/S_0$;
- sugar-to-product "i" yield coefficient ($Y_{i/S}$), the ratio between the produced mass of product "i" (cells or succinic acid) and the related decrease of the substrate mass.;
- SA specific productivity (P_{SA}), the ration between the SA concentration and the fermentation time.

Analytical methods

Cell density was measured as optical absorbance at 660 nm (OD_{660}) using a spectrophotometer (Cary- 50 Varian) and converted to dry biomass concentration. pH was also measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Dry cell mass (DCM) was determined from 10-mL samples centrifuged at 5000 rpm for 15 min at 4°C. Cell pellets were washed twice with distilled water and centrifuged between washes, then dried at 60 °C for 24 h under vacuum. The calibration procedure yielded: 1.0 OD_{660} was 0.377 g/L of DCM.

The concentration of soluble species was measured in the liquid phase after sample centrifugation (13000g, 10min). Sugar and organic acid concentration was measured by high performance liquid chromatography (HPLC) using a HP1100 chromatography working station system (Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9 µm; Bio-Rad Chemical Division, Richmond, CA), a UV absorbance detector (Agilent Technologies, G1315D), and a refractive index detector (Agilent Technologies, G1362A). H_2SO_4 5mM was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20 µL.

Results

Investigations were aimed to assess the ability of *A. succinogenes* to ferment sugars typically present in the hydrolysed fraction of lignocellulosic biomass and on the effects of some operating conditions on the process performance. The tested sugars included glucose, mannose, arabinose, and xylose. Two different initial sugar concentrations were investigated: 5 g/L and 40 g/L. The time resolved concentration of sugar and metabolites was measured.

Figure 3.1.1 reports data measured during a typical batch fermentation test carried out at initial glucose concentration of 40 g/L. In the graph are reported the pH and the concentration of cells growth, pH, sugar and the SA as a function of the time. The fermentation was stopped when the concentration of the metabolites were constant for more than 24 hours.

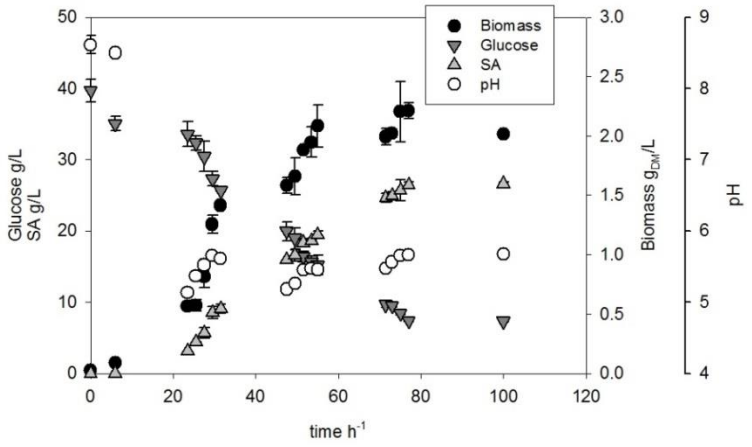

***Figure 3.1.1**: Time resolved concentration of biomass, glucose, SA, and pH measured during the A. succinogenes fermentation. Sugar: glucose. Carbone source initial concentration: 40 g/L.*

It should be mentioned that due to the presence of $MgCO_3$, the pH in the fermentation broth at the beginning was higher than 8. When the production of the acids started, the pH decreased to approach a constant value between 5 and 6.

Acetic acid and formic acid (data not shown) were also produced during the fermentation along with succinic acid, but at lower concentration. The final concentrations of the three acids produced are reported in Table 3.1.1.

***Table 3.1.1**: Final concentration of the acids produced during the fermentation carried out at initial glucose concentration of 40g/L*

	Final concentration, g/L
Succinic acid	26.6
Acetic acid	6.0
Formic acid	3.5

A. succinogenes had a similar growth pattern in all the fermentations and it was able to convert all sugars in acids. Figure 3.1.2 reports the time-series of sugar concentration and SA concentration measured during the fermentation tests carried out with the four investigated sugars at initial concentration set to 40 g/L. The fermentation lasted about three days. Except for xylose, no fermentation lag phase was observed. As regards xylose, about one day lag phase was observed. Figure 3.1.2A shows that: i) *A. succinogenes* was able to convert the investigated sugars; ii) the conversion degree depended on the sugar. Glucose was the sugar characterized by the best performances in terms of SA titer, SA yield and sugar conversion degree. The performances assessed for mannose, arabinose, and xylose fermentation did not depended on the sugar and they were lower than that assessed for glucose fermentation.

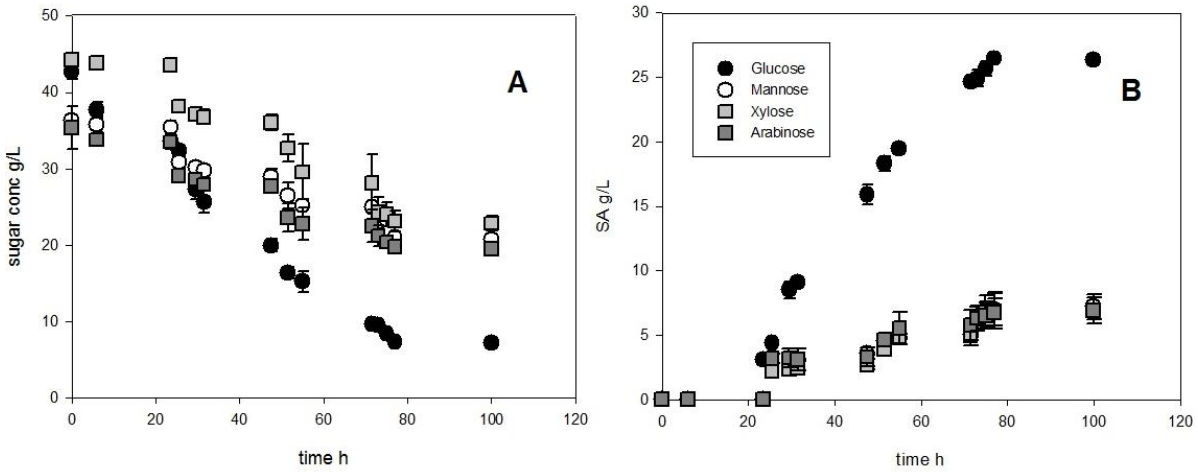

Figure 3.1.2: *Time resolved concentration of sugar (A) and succinic acid (B) measured during A. succinogenes fermentation. Carbone source initial concentration: 40 g/L*

Figure 3.1.3 reports the time-series of sugar concentration and SA concentration measured during the fermentations tests carried out with the four investigated sugars at initial concentration set at 5 g/L. The fermentation was almost completed after two days. In particular, the glucose fermentation ended for substrate depletion. No fermentation lag phase was observed for all sugars, It is worth to note that there were no significant differences in terms of sugar consumption and SA production profiles for the four sugars at this initial concentration. Glucose confirmed to be the sugar characterized by high performance even though the small differences.

Table 3.1.2 reports the results of all the fermentation tests. The SA selectivity was about constant in all the tests with values ranging between 0.50 and 0.58 g/g.

It is worth to note that for the lowest concentration investigated (5 g/L initial sugar) the performances in terms of sugar conversion degree, SA yield and productivity were comparable, indicating that there was not a strong sugar preference.

However, at initial sugar concentration set to 40 g/L, glucose was the sugar characterized by the best performances. In particular, glucose fermentation stopped when SA concentration approached a value of about 25 g/L while mannose, arabinose and xylose fermentation stopped when SA concentration approached a value of about 7 g/L, despite the sugar conversion was not complete whatever the sugar.

As it could be expected, the maximum SA productivity assessed for glucose fermentation increased with the initial concentration of the sugar as a result of the higher production rate and final SA concentration.

Moreover, the increase of the initial concentration of mannose, arabinose, and xylose from 5 to 40 g/L had only a slight effect on the maximum SA specific production. A possible explanation is that the average rate of the SA production increased as much as the SA concentration when the initial sugar concentration increased from 5 to 40 g/L.

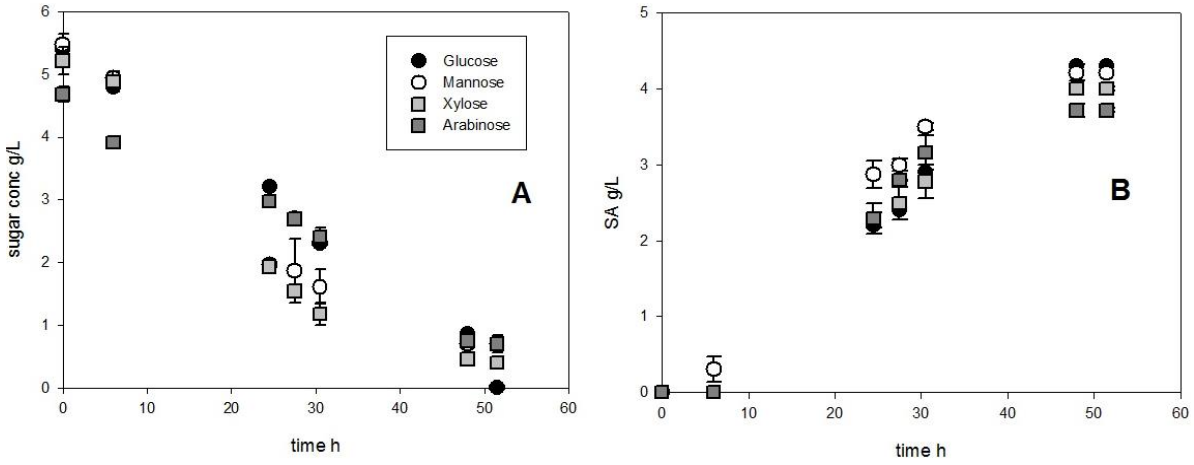

Figure 3.1.3: *Time resolved concentration of sugar (A) and succinic acid (B) measured during A. succinogenes fermentation. Carbone source initial concentration: 5 g/L.*

Table 3.1.2*: Main data regarding fermentation of A. succinogenes. The reported data were the value measured at the end of the fermentation tests. SA productivity (P_{SA}^{MAX}) was the maximum value assessed for each sugar.*

Initial sugar concentration, g/L	**Sugar**	**Conversion degree, ξ**	**SA g/L**	**$Y_{SA/S}$ g_{SA}/g_S**	**P_{SA}^{MAX} g_{SA}/L h**
5	Glucose	1	4.3	0.80	0.10
	Mannose	0.84	4.2	0.85	0.15
	Xylose	0.91	4.1	0.84	0.09
	Arabinose	0.84	4.0	0.86	0.10
40	Glucose	0.83	26.5	0.75	0.36
	Mannose	0.42	7.0	0.46	0.11
	Xylose	0.48	8.5	0.32	0.09
	Arabinose	0.44	6.8	0.43	0.13

Main remarks

The results obtained from fermentation tests on sugars typically present in the hydrolysed of lignocellulosic biomass pointed out that *A. succinogenes* was able of utilizing products of lignocellulosic biomass hydrolysates for the production of succinic acid. Glucose was the sugar characterized by the best performance when the initial concentration is set to 40 g/L. The performances are comparable for the four sugars investigated if the initial concentration is quite low (5 g/L). Further investigation on sugar mixtures -, with a composition that mime a typical lignocellulosic biomass hydrolysate, - will elucidate the synergic effects on the fermentation performances.

The kinetic characterization is also required for the design and optimization of a fermenter to produce succinic acid.

Acknowledgments

The study was supported by the Ministero dell'Istruzione, delll'Università e della Ricerca project "Development of green technologies for production of BIOchemicals and their use in preparation and industrial application of POLImeric materials from agricultural biomasses cultivated in a sustainable way in Campania Region – BIOPOLIS" PON03PE_00107_1/1.

3.2 Biosuccinic acid from lignocellulosic-based hexoses and pentoses by *Actinobacillus succinogenes*: characterization of the conversion process

Mariateresa Ferone, Francesca Raganati, Giuseppe Olivieri, Piero Salatino, Antonio Marzocchella*

Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale – Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

Abstract

Succinic acid (SA) is a well-established chemical building block. *Actinobacillus succinogenes* fermentation is by far the most investigated route due to very promising high SA yield and titre on several sugars. This study contributes to include the SA production within the concept of biorefinery of lignocellulose biomass. The study was focused on the SA production by *A. succinogenes* DSM 22257 using sugars representative from lignocellulose hydrolysis - glucose, mannose, arabinose and xylose - as carbon source. Single sugar batch fermentation tests and mixture sugar fermentation tests were carried out.

All the sugars investigated were converted in succinic acid by *A. succinogenes*. The best fermentation performances were measured in tests with glucose as carbon source. The bacterial growth kinetics was characterized by glucose inhibition. No inhibition phenomena were observed with the other sugar investigated.

The sugar mixture fermentation tests highlighted the synergic effects of the co-presence of the four sugars. Under the operating conditions tested, the final concentration of succinic acid in the sugar mixture test was larger (27 g/L) than that expected (25.5 g/L) by combining the fermentation of the single sugar. Moreover, the concentration of acetic and formic acid was lower, consequently obtaining an increment in the succinic acid specificity.

Keywords: Succinic acid, anaerobic fermentation, *Actinobacillus succinogenes*, lignocellulose, biorefinery

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Introduction

The limited nature of fossil reserves, the increasing worldwide demand for durable consumer goods and increasing environmental concerns are driving forces for a net reduction of the worldwide consumption of fossil resources. However, efficient biotechnological processes may contribute to the reduction of the consumption of fossil resources by converting renewable resources into chemical building-block.

Succinic acid (SA) is a well-established bio-based platform chemical (Bozell and Petersen, 2010). Succinic acid can be used as a feedstock chemical for the production of high value products such as 1,4-butanediol, tetrahydrofuran, adipic acid, γ-butyrolactone, and n-methylpyrrolidone, for applications in agriculture, food, medicine, plastics, cosmetics and textiles (McKinlay et al., 2007b; Zeikus et al., 1999). The global market of succinic acid has been estimated to be about 140'000 tonnes per year in 2014. Moreover, the market is expected to grow at an annual rate of 32.9% from 2010 to 2020 (GrandViewResearch, 2014). Nowadays, succinic acid is industrially produced by means of a petrochemical process from *n*-butane through maleic anhydride. Recently a biotechnological route has been proposed to produce succinic acid: the acid may be produced during microbial fermentations (Song and Lee, 2006). Bio-based succinate production may replace the petrochemical-based route with marked environmental benefits.

The capnophilic bacterium *Actinobacillus succinogenes* is considered one of the most promising microorganisms for industrial succinic acid production because of its ability to produce succinic acid at relatively high concentration. This Gram -negative anaerobic bacteria has been isolated from the bovine rumen. It has been proved that this bacterium is able to convert a wide spectrum of hexoses, pentoses, mono- and di-saccharides under anaerobic condition (Guettler et al., 1999). The metabolic pathway leading to SA production in *A. succinogenes* starts from the phosphoenolpyruvate (PEP) produced via the Embden-Meyerhof-Parnas pathway from glucose, with a little contribution of the pentose phosphate pathway (PPP) (McKinlay et al., 2007a). PEP is converted to SA via the reductive branch of the TCA cycle, and 1 mol of CO_2 is fixed per mol of SA produced. Indeed, it has been supposed that the CO_2 acts as a substrate for PEP carboxykinase (McKinlay et al., 2007a). As a result, the biologic production of succinic acid from both renewable resources and the greenhouse gas CO_2 would reduce the dependence of specialty chemical production on petroleum and the concentration of the CO_2 in the atmosphere.

The fermentation feedstock is a key factor affecting the cost of the succinic acid production. Potential feedstocks for the production of succinic acid by fermentation are lignocellulosic biomass. Indeed, some cheap biomass feedstocks such as whey, straw hydrolysate, and cane molasses have been reported for the production of bio-succinic acid (Kim et al., 2004; Wan et al., 2008; Zheng et al., 2009).

The present contribution regards the characterization of the succinic acid fermentation by *Actinobacillus succinogenes* DSM 22257 using sugars representative for the hydrolysis of lignocellulosic biomass: hexoses (glucose and mannose) and pentoses (arabinose and xylose). Batch tests were carried out to characterize the process in terms of succinic acid yield, sugar conversion degree and maximum SA concentration. The study included tests with single sugars and their mixtures to mime lignocellulosic hydrolysate composition. The assessment of interference-synergistic effects during the fermentation of the GMAX (glucose, mannose, arabinose, xylose) mixture has also been investigated.

Materials and Methods

Chemicals

All the chemicals used were of reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Microorganism

Actinobacillus succinogenes DSM 22257 supplied by DSMZ was used. Stock cultures were reactivated according to the method suggested by the supplier (DSMZ, n.d.) and stored in 20% glycerol at -80 °C.

The thawed cells were inoculated into 10 mL Brain Heart Infusion Broth medium in 15 mL Hungate tubes (pre-cultures). Vials were inoculated with a syringe from the headspace to ensure anaerobic conditions. The cultures were carried out for 24 h at 37°C and the agitation was provided by a rotary shaker (150 rpm).

Fermentation

The cells were grown in Pyrex screw capped anaerobic bottles sealed with a butyl rubber stopper under nitrogen atmosphere. The fermentation medium contained per litre: 5 g yeast extract, 1 g NaCl, 0.3 g Na_2HPO_4, 1.4 g NaH_2PO_4,1.5 g K_2HPO_4, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.23 g $CaCl_2 \cdot 2H_2O$. $MgCO_3$ as suspended solids at initial concentration of 5–30 g/L was supplied to the fermentation medium as an indirect CO_2 source and to buffer the pH during the cell growth. The medium was heat sterilized (20 min at 121◦C) prior to the carbon source addition. $MgCl_2$ and $CaCl_2$ were prepared as concentrated solutions (100×), sterilized by filtration and added aseptically to the autoclaved medium.

The carbon source investigated were representative of lignocellulosic hydrolysate: glucose, mannose, arabinose, and xylose (Ezeji and Blaschek, 2008). Concentrated stock solutions (300 g/L) were prepared for each investigated sugar. The sugar solutions were sterilized by filtration and supplemented to the autoclaved medium to the pre-set concentration. The initial concentration of the investigated sugars was set between 5 and 80 g/L.

Fermentation tests were also carried out with a sugar mixture made of glucose, mannose, arabinose, and xylose (GMAX mixture). The mass ratio between the sugars was set at 5:1:2:4, respectively, glucose/mannose/arabinose/ xylose (Raganati et al., 2015). The nominal total initial sugar concentration was set at 60 g/L.

The medium was inoculated with a 6.25 % (v/v) suspension of actively growing pre-cultures. Fermentations were carried out at 37°C without pH control and the agitation was provided by a rotary shaker (150 rpm). The cultures were sampled periodically to measure optical density, metabolites concentration and pH.

The batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and acids and solvents production. Measurements were processed to assess the following data:

- overall sugar conversion (ξ_S), i.e. the ratio between the sugar converted and the initial sugar $(S_0 - S)/S_0$;
- sugar-to-product “i” yield coefficient ($Y_{i/S}$), i.e., the ratio between the produced mass of product “i” (cells or succinic acid) and the related decrease of the substrate mass;

- the specific growth rate (μ). It was estimated at the beginning of the exponential phase as the slope of the biomass concentration (X) vs. time curve, on a log scale;
- the maximum specific succinic acid production rate (r^{max}_{SA}). It was estimated at the onset of the SA production as the slope of the SA concentration vs. time curve, divided by the cells concentration measured;
- the SA selectivity with respect to the other produced acids (χ_{SA}). It was the ratio between the SA concentration and the concentration of all produced acids;
- SA productivity (P_{SA}). It was estimated as the ratio between the produced succinic acid and the time.

Each test was carried out in triplicate and the mean values are reported as results. The error was typically within 5 %.

Analytical methods

Cell density was measured as optical absorbance at 660 nm (OD_{660}) using a spectrophotometer (Cary- 50 Varian). pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments).

The dry cell weight (DW) was determined following this procedure: 10-mL samples of the cultures were centrifuged at 4°C for 15 min at 5000 rpm; cell pellet was washed twice with distilled water, then the recovered washed pellet was dried at 60 °C for 24 h under vacuum and weighted (Maharaj et al., 2014). The regression of OD and weight data provided OD conversion factor into cell dry concentration: 0.38 g_{DW} L^{-1}/OD_{660}.

After sampling, the biomass was centrifuged at 13,000g for 10min. Sugar and organic acid concentration were measured by high performance liquid chromatography (HPLC) using a HP1260 chromatography working station system (Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9 μm; Bio-Rad Chemical Division, Richmond, CA), UV absorbance (Agilent Technologies, G1315D) and refractive index detector (Agilent Technologies, G1362A). 5mM H_2SO_4 was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20 μL.

Growth rate calculation

Kinetic data were interpreted according to unsegregated-unstructured models: the classical hyperbolic Monod model and a model characterized by substrate inhibition were used. The equations were:

Monod model: $\mu = \mu_{max} * \left(\frac{S}{S+K_M}\right)$ (3.2.1)

Haldane model: $\mu = \mu_{max} * \left(\frac{S}{S+K_M+{S^2}/{K_i}}\right)$ (3.2.2)

where μ is the specific growth rate (h^{-1}), μ_{max} (h^{-1}), K_M (g_S/L) and K_i (g_S/L) are constants, and S the substrate concentration (g_S/L).

The inhibitory effects of the produced acids were not taken into account because the specific growth rate was estimated at the beginning of the exponential phase. Under these conditions the concentration of the acids (SA, AA and FA) was below the detection limit or more then 10-fold lower the inhibitory concentration reported (Lin et al., 2008b).

Results and Discussion

Batch fermentation of single sugar

The activity was aimed to assess the feasibility of *A. succinogenes* to ferment sugars typically present in the hydrolysed fraction of lignocellulosic biomass. The batch fermentation tests were carried out in the standard medium supplemented with the investigated sugars - glucose, mannose, arabinose, and xylose - as carbon source.

The experimental data reported in Fig. 3.2.1 refer to a batch fermentation test carried out at initial glucose concentration of 40 g/L. Figure 3.2.1 reports the time-resolved measurements of the concentration of *A. succinogenes* cells, glucose, and metabolites (succinic acid, acetic acid, formic acid) as well as of pH. No lag phase was observed under reported conditions and, as expected, the exponential cell growth phase was coupled with fast consumption of the sugar and the simultaneous production of the three acids (SA, AA and FA). At about 30 h, the cell growth stopped, the glucose consumption rate decreased and the AA and FA concentration approached a constant value. The fermentation furtherly proceeded until about 80 h: glucose conversion was up to about 80% and, SA was continuously produced up to concentration of about 27 g/L. The fermentation test was stopped when the concentration of cells, glucose, and metabolites were constant for more than 24 hours. The pH in the fermentation broth was higher than 8 at the beginning for the presence of $MgCO_3$, and it decreased during the fermentation for the production of acids to approach a constant value between 5 and 6.

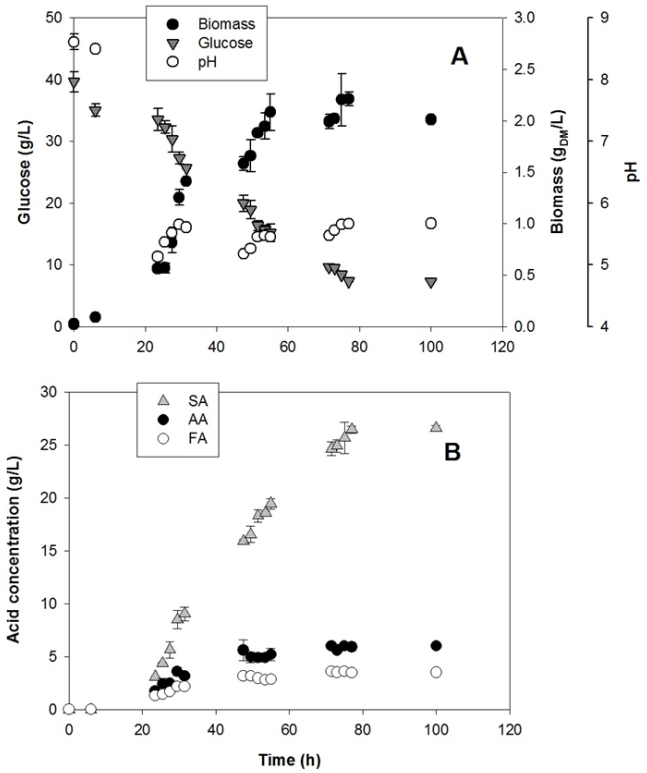

Figure 3.2.1: *Time resolved measurements carried out during the A. succinogenes fermentation. Sugar: glucose. Carbone source initial concentration: 40 g/L. a) pH and concentration of biomass and glucose. b) concentration of SA, AA and FA.*

Figure 3.2.2 reports the time-series of sugar concentration and SA concentration measured during the fermentation tests carried out with the four investigated sugars at

initial concentration set to about 40 g/L. The data for glucose reported in Figure 3.2.1 were also included in the Figure 3.2.2 to have a full view of the phenomena. The fermentation lasted about five days for all sugars.

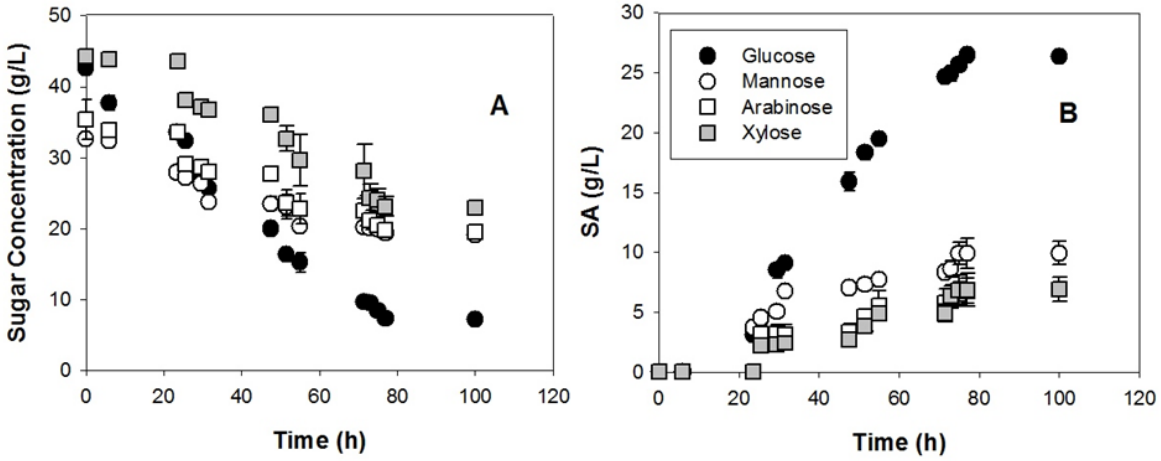

***Figure 3.2.2**: Data measured during batch fermentations of A. succinogenes in standard medium supplemented with the investigated sugars Nominal initial concentration of the investigated sugar: 40 g/L. a) Residual sugar concentration. b) SA concentration*

A. succinogenes fermentation path did not change with the investigated sugar: cells were able to convert all sugars in acids. The SA production continued in all fermentations even though cells were under stationary growth phase. It is interesting to compare the amount of SA produced under growth and no-growth phase. For the test carried out at 40 g/L of initial glucose concentration, the SA produced under the exponential phase was about 16 g/L and that produced under no-growth phase is about 11 g/L. The SA production without cell growth is in agreement with previous investigation carried out under batch conditions (Corona-Gonzalez et al., 2010; Du et al., 2007; McKinlay et al., 2007a). The SA production under the two phases is comparable and it confirms that production of SA under no-growth conditions plays an important role in the SA production. It should be noted that the SA production without cell growth is typically no lower than that under growth conditions. Therefore, a culture kept successfully under no-growth conditions is a potential producer of SA.

In Table 3.2.1 are reported the most relevant data of the batch fermentation tests carried out with investigated sugars at initial concentration of about 40 g/L. The analysis of Figure 3.2.2 and of the data reported in Table 3.2.1 points out that: i) *A. succinogenes* was able to grow on the investigated sugars; ii) the conversion degree depended on the sugar; iii) the fermentation performance expressed in terms of succinic acid concentration and production yield depended on the sugar. Glucose was the best metabolized sugar and it was characterized by the highest SA concentration. The performance assessed for mannose, arabinose and xylose fermentations were lower than that assessed for glucose fermentation and were not dependent on the substrate concentration.

***Table 3.2.1**: Main results of batch fermentation tests using single sugar as carbon source*

Sugar initial conc. g/L	ξ_S -	SA titer g/L	$Y_{SA/S}$ g_{SA}/g_{GLU}	χ_{SA} g_{SA}/g_{Ac}	P_{SA}^{max} $g_{SA}/L*h$	Growth $Y_{SA/S}$ g_{SA}/g_{GLU}	No-Growth$Y_{SA/S}$ g_{SA}/g_{GLU}
Glucose							
5.4	1.00 ±0.00	4.3 ±0.3	0.80 ±0.09	0.56 ±0.01	0.10 ±0.02	1.00 ±0.03	0.66 ±0.05
21.8	0.94 ±0.09	14.0 ±1.1	0.69 ±0.10	0.58 ±0.00	0.20 ±0.00	0.65 ±0.06	0.70 ±0.02
42.7	0.83 ±0.01	26.5 ±0.4	0.75 ±0.03	0.74 ±0.01	0.36 ±0.01	0.53 ±0.04	0.95 ±0.03
67.5	0.47 ±0.01	18.6 ±0.2	0.58 ±0.01	0.65 ±0.00	0.29 ±0.03	0.49 ±0.03	0.70 ±0.04
80.7	0.28 ±0.01	14.6 ±1.1	0.65 ±0.04	0.62 ±0.01	0.22 ±0.01	0.43 ±0.02	1.10 ±0.06
Mannose							
5.7	0.84 ±0.06	4.0 ±0.1	0.86 ±0.04	0.55 ±0.01	0.15 ±0.06	0.83 ±0.04	0.91 ±0.03
9.6	1.00 ±0.00	5.0 ±0.2	0.53 ±0.03	0.53 ±0.01	0.15 ±0.00	0.54 ±0.03	0.46 ±0.01
15.9	0.56 ±0.02	5.8 ±0.1	0.65 ±0.02	0.47 ±0.02	0.13 ±0.03	0.65 ±0.02	0.65 ±0.02
20.7	0.49 ±0.04	5.0 ±0.2	0.50 ±0.00	0.44 ±0.01	0.10 ±0.00	0.52 ±0.01	0.47 ±0.03
32.6	0.41 ±0.01	9.9 ±0.2	0.75 ±0.05	0.46 ±0.02	0.17 ±0.04	0.82 ±0.05	0.71 ±0.06
Arabinose							
5.0	0.84 ±0.01	4.0 ±0.4	0.96 ±0.03	0.58 ±0.04	0.11 ±0.00	1.10 ±0.02	0.82 ±0.03
10.6	1.00 ±0.00	3.7 ±0.5	0.35 ±0.01	0.41 ±0.05	0.09 ±0.01	0.34 ±0.03	0.36 ±0.05
15.9	0.46 ±0.09	6.9 ±1.1	0.93 ±0.08	0.57 ±0.03	0.13 ±0.02	1.12 ±0.04	0.76 ±0.07
19.5	0.55 ±0.05	6.8 ±0.2	0.60 ±0.03	0.56 ±0.01	0.20 ±0.05	0.59 ±0.03	0.70 ±0.02
35.0	0.44 ±0.03	6.8 ±1.1	0.44 ±0.13	0.50 ±0.04	0.13 ±0.01	0.42 ±0.01	0.45 ±0.04
Xylose							
5.2	0.91 ±0.01	4.0 ±0.00	0.84 ±0.02	0.56 ±0.02	0.09 ±0.02	0.68 ±0.02	1.00 ±0.04
12.8	0.63 ±0.13	5.1 ±0.42	0.63 ±0.11	0.56 ±0.00	0.10 ±0.02	0.52 ±0.09	0.83 ±0.02
21.3	0.83 ±0.02	8.2 ±0.35	0.43 ±0.03	0.48 ±0.01	0.11 ±0.00	0.32 ±0.03	0.60 ±0.05
44.3	0.64 ±0.03	13.7 ±0.63	0.48 ±0.00	0.61 ±0.02	0.09 ±0.02	0.36 ±0.06	0.52 ±0.03
53.9	0.22 ±0.01	4.2 ±0.01	0.35 ±0.02	0.51 ±0.02	0.09 ±0.00	0.50 ±0.04	0.23 ±0.10

Succinic acid yield ($Y_{SA/S}$) depended on the sugar. In particular, the SA yield is about 0.75 g of SA per gram of sugar for the hexose sugars (glucose and mannose), and it is about 0.48 and 0.43 for xylose and arabinose, respectively. Despite the same value of yield measured for glucose and mannose, the final SA concentration in tests with mannose is 2.5-fold smaller that measured in the tests with glucose. Of course, the lower sugar conversion measured for mannose implies a lower SA concentration.

The yields obtained in this work are generally higher if compared to similar studies in which succinic acid production at different sugars concentration was investigated (Jiang et al., 2013; Pateraki et al., 2016; Zheng et al., 2009). However, in some cases the yield decrease with increasing initial sugar concentration; this trend might be attributed to the higher maintenance requirements at low sugar concentration that leads to higher production of succinic acid and less biomass production.

The ability of *A. succinogenes* to metabolize a wide spectrum of carbon sources is in part due to the rumen, the *A. succinogenes*'s natural habitat where a variety of carbohydrate substrates are available (Samuelov et al., 1991). However, the carbon source up-take and conversion mechanism depend on the sugar. Glucose and mannose are taken up by a phosphotransferase system (PTS), arabinose and xylose are taken up by an ATP-dependent transporter; xylose can also be transported by a H+-symport

mechanism involving a separate transporter. Glucose-6-phosphate is catabolized to phosphoenolpyruvate (PEP) via glycolysis or via the pentose phosphate pathway (PPP), even if in minor part. Mannose-6-phosphate is isomerized to fructose-6-phosphate and enters both the glycolytic and the PPP. Arabinose and xylose are isomerized, respectively, to ribulose and xylulose, which are metabolized according to the PPP (McKinlay et al., 2010). The different mechanisms of up-take and degradation pathway for the four sugars could explain the differences in the conversion degree and consequently of the SA production from each sugar.

Effect of the sugar on growth rate

To study the effect of the sugar on *A. succinogenes* growth and succinic acid production, fermentations were carried out at different initial sugar concentrations (mean of at least three replicas). Main results of the batch tests at different initial sugar concentration are reported in Table 3.2.1.

Fig. 3.2.3 presents experimental and model results for the specific growth rate as a function of initial sugar concentration. Values of specific growth rates were determined for each initial sugar concentration by plotting ln(X) versus time (results not shown).

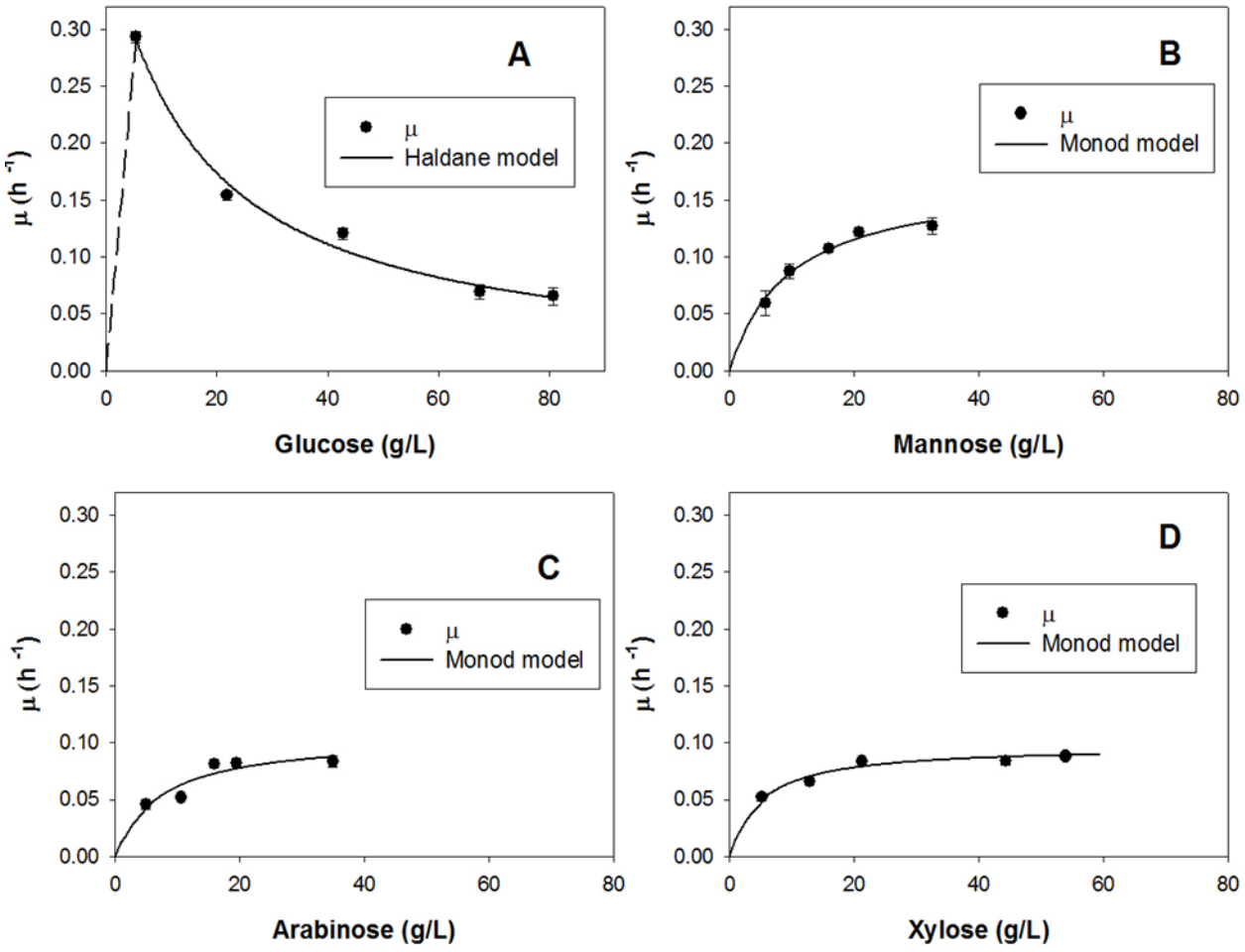

Figure 3.2.3: *Experimental data and simulation results of specific growth rate for glucose, mannose, arabinose and xylose using the proposed models.*

Figure 3.2.3 reports the specific growth rates versus the initial sugar concentration for each sugar investigated. This Figure also shows the plots of the expected value of each specific growth rate assessed according the Eqs. (3.2.1) and Eqs. (3.2.2).

A. succinogenes growth rate was characterized by glucose inhibition (Fig.3.2.3 A) within the investigated range of concentration. Several studies (Corona-González et al., 2008; Van der Werf et al., 1997)resulted in the same behavior and proposed a kinetic model to characterize cell growth, substrate consumption and product formation in *A. succinogenes* fermentations. They pointed out that substrate and product inhibition have a significant effect on cell growth and succinic acid production. However, in this study the inhibitory effects of the acids was not taken into consideration because the specific growth rate was estimated at the beginning of the exponential phase, when the concentration of the acids (SA, AA and FA) was more then 10-fold lower the inhibitory concentration reported (Lin et al., 2008a).

As regard the other sugar investigated-xylose, mannose and arabinose- no inhibition phenomena were observed on *A. succinogenes* growth. Therefore, the Monod model was used to fit the experimental data and calculate the kinetic constants. In Table 3.2.2 are reported the kinetic parameters estimated using the proposed models. The main observation are:

- The maximum specific growth rate is definitively higher for glucose then the other sugars;
- *A. succinogenes* growth is characterized by glucose inhibition, but no inhibition phenomena are observed with the other sugars;
- The constant K_M – the concentration of limiting substrate at which the specific growth rate is half the maximum growth rate – are in the range 4.8-9.5 g/L in the order: xylose<arabinose<mannose;
- The inhibition constant K_i for glucose is 15.5 g/L

The values of correlation coefficients (R^2) were higher than 0.95, except for arabinose, confirming that the models accurately represented the experimental data.

Table 3.2.2: *Kinetic parameters assessed by processing experimental data according to the proposed models.*

Sugar	**Model**	**μ_{max} h^{-1}**	**K_M g/L**	**K_i g/L**	**R^2 -**
Glucose	Haldane	0.39 ±0.02	0.1 ±0.01	15.45 ±0.3	0.99
Mannose	Monod	0.17 ±0.01	9.49 ±0.71	-	0.98
Xylose	Monod	0.10 ±0.01	4.82 ±0.81	-	0.95
Arabinose	Monod	0.11 ±0.02	7.14 ±0.34	-	0.82

The specific growth rate values obtained in this study are lower than those reported in literature (Corona-González et al., 2008; Lin et al., 2008a) for this strain. This behavior finds explanation in the operating conditions, since pH was not controlled during fermentation. Van der Werf et al.(Van der Werf et al., 1997) reported that cell growth was affected adversely at low pH, possibly reflecting higher maintenance requirements at lower pH values.

The maximum SA productivity was 0.36 g/L*h and it was assessed for tests carried out with glucose as carbon source at initial concentration of 42.7 g/L. The productivity assessed from the test carried out with the other sugar investigated was generally lower that that obtained on glucose. This comparison confirms that the glucose is the sugar characterized by the highest fermentation performance in terms of productivity and yield. However, for initial sugar concentration lower than 20 g/L the SA

productivity measured during the tests does not depend remarkably on the (investigated) sugar.

Fermentation of sugar mixture

Fermentation tests were carried out with a mixture of the four investigated sugars (glucose/mannose/arabinose/xylose - GMAX) to assess the possible synergic/interfering effects. The nominal total initial sugar concentration was set at 60 g/L and the mass ratio between the sugars was set at 5:1:2:4 for the GMAX mixture (Ezeji and Blaschek, 2008; Raganati et al., 2015).

The Figure 3.2.4 reports the time resolved concentration of the sugars and of acids measured during the fermentation with the GMAX mixture. All the sugars were simultaneously converted during the fermentation even though the rate of sugar uptake was sugar specific. This is in agreement with similar works (Jiang et al., 2013; Zheng et al., 2009), were a simultaneous conversion of different sugar was observed. No diauxic growth was observed. As for the single sugar tests, the concentration of succinic acid continuously increased with the sugars conversion up to about 27 g/L, while acetic and formic acid approached a constant value after about 50 hours of fermentation. The sugar preference was not obvious when they were mixed. Glucose was not confirmed as the preferred substrate for *A. succinogenes.* Indeed, the initial specific sugar conversion rate (r^0_S) for each sugar of the mixture suggests that the order of sugar preference was: xylose>arabinose>glucose>mannose. In other words, *A. succinogenes* preferred pentose sugars over hexoses sugars when they were in mixtures. The preference is in contrast with the observation made in the batch test on single sugars where glucose was the preferred sugar.

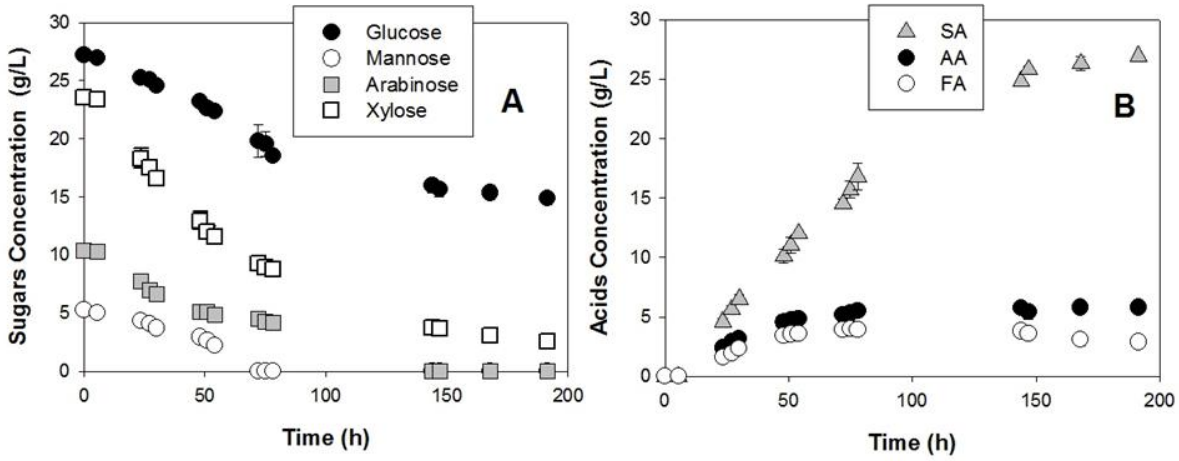

Figure 3.2.4: *Time resolved concentration of a) sugars and b) acids (SA, AA and FA) measured during the A. succinogenes batch fermentation on GMAX mixtures.*

Table 3.2.3 summarizes main data regarding the fermentation tests carried out with GMAX mixtures. A total of 27.0 g/L succinic acid was obtained, a productivity of 0.22 g/L*h and a yield of 0.55 g_{SA}/g_S. Although the succinic acid concentration is about that measured in the glucose control test at the same overall concentration (Tab. 3.2.2), the fermentation times was longer (200 h) than the control (100 h) resulting in a lower productivity. Similar results, in terms of succinic acid concentration and yield were obtained by Jiang et al.(Jiang et al., 2013); they used a different sugar mixture for batch fermentation, which was composed of cellobiose, glucose, arabinose and xylose,

obtaining 21.7 g/L of succinic acid and a yield of 0.68 $g_{SA}/g_{S.}$ However they also had higher concentration of acetic acid (about 15 g/L) as side product compared to this work (about 6 g/L), thus reducing the specificity.

***Table 3.2.3**: Fermentations of A. succinogenes using sugar mixtures as carbon source. Nominal total initial concentration of sugars: 60 g/L.*

ξ_S	
Glucose	0.45 ± 0.02
Mannose	0.90 ± 0.00
Arabinose	1.00 ± 0.00
Xylose	1.00 ± 0.00
Tot sugars	0.74 ± 0.01
Final acids concentration	g/L
SA	27.00 ± 0.07
AA	5.80 ± 0.14
FA	2.90 ± 0.14
χ_{SA}	0.75 ± 0.00
Yields	g/g_S
$Y_{SA/S}$	0.55 ± 0.01
$Y_{AA/S}$	0.06 ± 0.00
$Y_{FA/S}$	0.12 ± 0.00
r^{MAX}_{SA}	0.35 ± 0.07 g_{SA}/g_{DM} h
r^0_S	g_S/g_{DM}*h
Glucose	0.14 ± 0.02
Mannose	0.08 ± 0.03
Arabinose	0.22 ± 0.01
Xylose	0.41 ± 0.02
μ	0.14 ± 0.00 h^{-1}
$P_{SA}{}^{max}$	0.22 ± 0.00 g_{SA}/L*h

Succinic acid fermentation from sugar mixtures was also reported by Li et al. (Li et al., 2010b). The sugar mixtures, derived from the hydrolysis of corn stalks, contained mainly glucose, xylose and cellobiose; with this feedstock they obtained 17.8 g/L of succinic acid, corresponding to a yield of 0.66 g_{SA}/g_S. In both these studies higher productivities were achieved (0,66 g/L*h for both), since the fermentation times was shorter, respectively 35h and 40h.

The χ_{SA} ratio was higher in the GMAX tests (0.74) with respect to the single sugar tests (values ranging between 0.45 and 0.60 in most of the tests).

To assess the synergic effects of the sugars in the GMAX mixture, the expected values of acid concentration were calculated according to the following relationship:

$$\overline{Ac} = \sum_{sugar_i} Y_{Ac/S} \, (S_i^0 - S_i) \qquad (3.2.3)$$

where Ac is the concentration of a generic acid (SA, AA, and FA). The Table 3.2.4 reports the comparison between the expected and the measured concentration of the acids. The measured SA concentration was higher than the expected value, while the measured concentration of AA and FA were lower. The SA selectivity benefits from the co-presence of the four sugars in the fermentation medium.

Table 3.2.4: *Expected and experimental acid concentrations for the GMAX fermentation test.*

	SA (g/L)	AA (g/L)	FA (g/L)
Expected	25.5	16.0	11.8
Experimental	27.0	5.8	2.9

Conclusions

Anaerobic batch fermentation for the succinic acid production by *A. succinogenes* from different sugars has been studied.

Tests with single sugars pointed out that *A. succinogenes* growth rate is affected by inhibition by glucose while no inhibition phenomena were observed with mannose, arabinose and xylose. The reported results have to be integrated with kinetic characterization of the bioconversion process and continuous tests will be also necessary for the assessment of kinetics and yields.

Tests with mixtures pointed out that: i) *A. succinogenes* was able to simultaneously metabolize the investigated sugars (glucose, mannose, arabinose, and xylose); ii) the fermentation took advantage of the co-presence of the four investigated sugars with respect to the tests carried out with single sugars. The synergistic effect of the four sugars led to an increase in succinic acid concentration and SA selectivity.

Acknowledgments

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3.3 Efficient succinic acid production from leftover beverages by *Actinobacillus succinogenes*

Mariateresa Ferone, Alessia Ercole, Giuseppe Olivieri, Piero Salatino, Antonio Marzocchella

Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale - Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

Abstract

In the present contribution, high-sugar- content beverages (HSCBs) were used as a renewable feedstock to produce SA by *Actinobacillus succinogenes*. The aims of this study are: (1) to identify an alternative disposal process for the industry of HSCBs and (2) to study succinic acid (SA) production from non-edible feedstocks. Three types of commercial beverages were investigated: fruit juices (pineapple and ace), syrup (almond) and soft drinks (cola and lemon). They contained high concentration of sugars—between 50 and 1,000 g/L—mainly glucose, fructose, and sucrose. These sugars were also tested individually and in a mixture. The batch fermentation tests highlighted that *A. succinogenes* was able to grow on HSCBs supplemented with yeast extract, but also on the unsupplemented fruit juices. The HSCB pre-hydrolysis improved the fermentation performance in terms of succinic acid production and sugar conversion.

Keywords: Succinic acid, *Actinobacillus succinogenes*, high-content-sugar beverages, biorefinery, fermentation.

Introduction

Over the last decades, there has been a growing interest in producing chemicals from renewable raw and waste materials in order to reduce the environmental and economic issues related to the petroleum-derived chemicals production (Becker et al., 2015).

Succinic acid (SA), a four carbon aliphatic dicarboxylic acid, was identified in 2004 as one of the top value-added chemicals produced from biomass by the US Department of Energy (DOE) (Werpy and Petersen, 2004). It can find application in a variety of industrial fields, from high-value applications such as personal care products, pharmaceutical and food additives, to large volume applications such as plasticizers, polyurethanes, resins, coatings and chemical intermediates (Litsanov et al., 2014).

Actinobacillus succinogenes, a capnophilic Gram negative bacteria isolated from the bovine rumen, is considered one of the most promising microorganisms for industrial succinic acid production (Guettler et al., 1999). This bacterium has the ability to produce succinic acid at relatively high concentration (Guettler, 1996) and is able to convert a wide spectrum of hexoses, pentoses, mono- and di-saccharides under anaerobic condition (Guettler et al., 1999).

The production of bio-based succinic acid from renewable materials has been researched for several years and innovative production routes are still being discovered and optimized. However, at present, the fermentative production of succinic acid forms only a small share of the total production of succinic acid worldwide and most of the commercially available succinic acid is produced through the chemical conversion of maleic anhydride, derived from fossil resource (GrandViewResearch, 2014).

An economically feasible bio-succinic acid production requires low-cost raw materials which can be supplied continuously in large quantities as a feedstock. The feedstock cost could be drastically reduced if waste materials are used. Several typologies of waste(water)s have already been reported for the production of bio-succinic acid (Carvalho et al., 2016; Scholten et al., 2009; Wan et al., 2008).

In this study, another non-conventional substrate was evaluated as a source for sugars for biofuels and biochemical production. Potential feedstock for succinic acid production are High-sugar-content beverages (HSCBs), such as fruit juices, syrups, soft drinks and sport drinks. The use of HSCBs offers many advantages. First of all, they contain high concentration of simple sugars (glucose, fructose, sucrose) that can be fermented by a vast majority of the commonly utilized microbial strains without any need for hydrolysis.

In many cases they can represent food industry waste (process wastewaters, seizures of badly repaired or illegal goods, and stocks after their expiration date). The disposal of waste beverages is then a critical issue for the industry because of cost of the treatment (up to 500€/m^3) due to the high Chemical Oxygen Demand (COD) and the suspended solid concentration. The European HSCBs market size is of the order of 70 Mtonn per year and 0.1% can be taken into account as the stream to be disposed or remediated (Procentese et al., 2016).

Another advantage for these kind of waste is that some beverage (especially soft drink) contain high carbon dioxide concentration, which minimizes the diffusion of the oxygen. Therefore, they can be added directly to the reactor without need for pre-treatment or purging with an inert gas, like nitrogen or argon, to remove the residual

oxygen. This is important as the fermentative strains are generally either aero-tolerant anaerobic strains, such as *Zymomonas mobilis* (He et al., 2014), or strict anaerobes, like *Clostridia* strains (Diekert and Thauer, 1978; Monot et al., 1982).

Few examples of biofuels production from beverages are present in literature. Dwidar et al. (Dwidar et al., 2012) used carbonated beverages as carbon source to produce ethanol with *Zymomonas mobilis* and butanol by means of three different clostridial strains. Raganati et al. (Raganati et al., 2014) suggested to use leftover beverages as a carbon source for butanol production with *Clostridim acetobutylicum*.

To the authors' knowledge, a thorough characterization of succinic acid production from high-sugar-content beverages is still absent in the literature.

In this paper, fermentative production of succinic acid by *Actinobacillus succinogenes* from leftover beverages was studied. Three types of commercial beverages were tested: fruit juices (pineapple and ace), syrup (almond) and soft drinks (cola and lemon). The sugar concentration of the beverages investigated ranged between 100 and 1,000 g/L (see Table 3.3.1). The fermentations were characterized in terms of overall sugar conversion, maximum succinic acid concentration, yield, and productivity.

Materials and Methods

Microorganism

Actinobacillus succinogenes DSM 22257 supplied by DSMZ was used. Stock cultures were reactivated according to the method suggested by the supplier ("www.dsmz.de (accessed: 15.12.2014)) and stored in 20% glycerol at -80 °C. The thawed cells were inoculated into 12 mL Brain Heart Infusion Broth medium in 15 mL Hungate tubes (pre-cultures). Vials were inoculated with a syringe from the headspace to ensure anaerobic conditions. The cells were grown for 24 h at 37°C and the agitation was provided by a rotary shaker (150 rpm). Then, the pre- cultures were transferred into the fermentation bottles.

Medium

Synthetic Medium Supplemented with Glucose, Fructose, and Sucrose as a Carbon Source

The synthetic fermentation medium contained: 5 g/L yeast extract (YE), 1 g/L NaCl, 0.3 g/L Na_2HPO_4, 1.4 g/L NaH_2PO_4,1.5 g/L K_2HPO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.23 g/L $CaCl_2 \cdot 2H_2O$. $MgCO_3$ as suspended solids at initial concentration of 5–30 g/L was supplied to the fermentation medium as an indirect CO_2 source and to buffer the pH during the cell growth. The medium was heat sterilized (20 min at 121°C) prior to the carbon source addition. $MgCl_2$ and $CaCl_2$ were prepared as concentrated solutions (100×), sterilized by filtration and added aseptically to the autoclaved medium.

The carbon source investigated were the sugar typically present in the investigated beverages: glucose, fructose and sucrose (Raganati et al., 2014). Concentrated stock solutions (300 g/L) were prepared for each investigated sugar. The sugar solutions were sterilized by filtration and supplemented to the autoclaved medium to the pre-set concentration.

High-sugar content beverages

Table 3.3.1 lists the tested HSCBs characterized in terms of pH, sugars and protein content and brand. The data refer to commercial beverages close to the

expiration data. They were selected among the most popular beverages: two fruit juices, a concentrated syrup (used to prepare drinks) and two soft drinks. The juices and the soft drinks were diluted, 1:2 and the syrup was diluted 1:20, respectively, before fermentation. For all the beverages, the pH was adjusted at pH=7 and then it was sterilized in autoclave (120 °C for 20 min).

The fermentation tests were carried out with both HSCB supplemented with nutrients (HSCB+) and HSCB unsupplemented (HSCB-). In the supplemented HSCB were added all the medium components listed in the section 2.2.1; in the usupplemented HSCB only $MgCO_3$ was added, in order to provide an inorganic CO_2 source to produce succinic acid.

HHSCB+ tests: Hydrolyzed HSCBs were also investigated. Hydrochloric acid was added to the HSCB until pH=1.5 was reached and then the solution was autoclaved. Apart from the carbon source, the composition of the medium was the same as for the HSCB+ tests.

Table 3.3.1: *High-sugar-content beverages investigated: pH, sugars and protein concentration, brand*

HSCB	pH	Sugar concentration g/L	Protein concentration g/L	Brand
Pineapple juice	2.5-3	120	4.0	Rauch Bravo
ACE juice	2.5-3	110	2.0	Rauch Bravo
Cola	2.5-3	120	-	Coca- Cola Company
Sprite	2.5-3	100	-	Coca- Cola Company
Almond syrup	5.5-6	1000	-	Toschi Vignola

Fermentation

The cells were grown in Pyrex screw capped anaerobic bottles sealed with a butyl rubber stopper under nitrogen atmosphere.

The medium was inoculated with a 6.25 % (v/v) suspension of actively growing pre-cultures. Fermentations were carried out at 37°C without pH control and the agitation was provided by a rotary shaker (150 rpm). The cultures were sampled periodically to measure optical density, metabolites concentration and pH.

The batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and acids and solvents production. Measurements were processed to assess the following data:

- overall sugar conversion (ξ_S), i.e. the ratio between the sugar converted and the initial sugar $(S_0 - S)/S_0$;
- sugar-to-product “i” yield coefficient ($Y_{i/S}$), i.e., the ratio between the produced mass of product “i” (cells or succinic acid) and the related decrease of the substrate mass;
- the SA selectivity with respect to the other produced acids (χ_{SA}). It was the ratio between the SA concentration and the concentration of all produced acids;
- SA productivity (W_{SA}). It was estimated as the ratio between the produced succinic acid and the time.

Analytical methods

Cell density was measured as optical absorbance at 660 nm (OD_{660}) using a spectrophotometer (Cary- 50 Varian). pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments).

The dry cell weight (DW) was determined as previously described (M. Ferone et al., 2017b). The regression of OD and weight data provided OD conversion factor into cell dry concentration: 0.38 g_{DW} L^{-1}/OD_{660}.

After sampling, the biomass was centrifuged at 13,000g for 10min. Sugar and organic acid concentration were measured by high performance liquid chromatography (HPLC) using a HP1260 chromatography working station system (Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9 µm; Bio-Rad Chemical Division, Richmond, CA), UV absorbance (Agilent Technologies, G1315D) and refractive index detector (Agilent Technologies, G1362A). 5mM H_2SO_4 was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20 µL.

Results and Discussion

Fermentation of synthetic SGF medium

Prior to carrying out HSCB fermentation, fermentation of synthetic media containing sugars (glucose, sucrose and fructose) typically present in HSCB was carried out, both to verify the ability of *Actinobacillus succinogenes* DSM 22257 to metabolize them and to establish a reference for evaluation of its performance on HSCB.

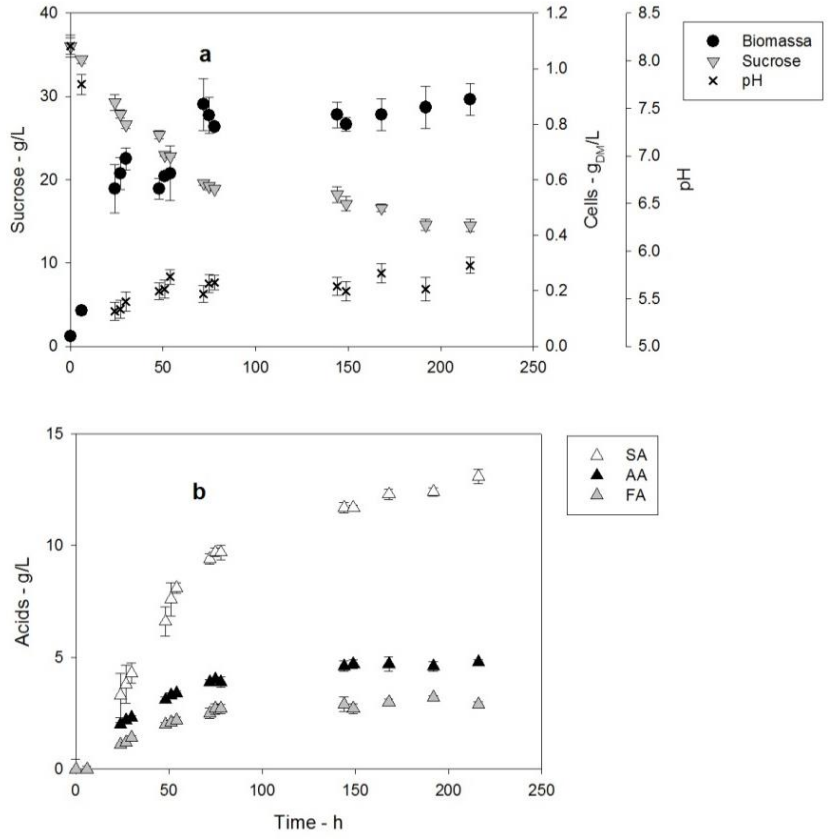

Figure 3.3.1: *Data measured during A. succinogenes fermentation in standard medium supplemented with sucrose (30 g/L); a) concentration of biomass and sugar b) pH and concentration of SA, AA and FA.*

Figure 3.3.1 reports the time-resolved profiles of the concentration of *A.succinogenes* cells, sucrose (initial concentration 30 g/L), and produced acids (succinic, acetic and formic acid) as well as of pH, measured during a batch culture. As expected, the exponential cell growth phase was coupled with fast consumption of the

sugars and the simultaneous production of the three acids (SA, AA and FA). At about 70 h, the cells entered the stationary phase of growth, the sugar was consumed at lower rate and the AA and FA concentration approached a constant value. The fermentation furtherly proceeded until about 250 h; the test was stopped when the concentration of cells, sucrose and metabolites were constant for more than 24 hours. Sucrose was not completely consumed: about 15 g/L of the sugar remained unconverted.

***Table 3.3.2**: Relevant data of A. succinogenes fermentation tests carried out using glucose (G), fructose (F), and sucrose (S) as carbon source*

	ξ	SA g/L	$Y_{SA/S}$ g_{SA}/g_S	χ_{SA} g/g	W_{SA} g/L*h
Glucose	0.94	14.0	0.68	0.58	0.18
Fructose	0.70	13.3	0.67	0.62	0.10
Sucrose	0.70	13.1	0.61	0.63	0.10
SGF	0.95	40.3	0.68	0.82	0.19

The experimental data reported in Figure 3.3.2 refer to a batch fermentation test where sucrose, glucose and fructose (SGF) were added in mixture at the sugar ratio set to 1:1:1 (total sugars concentration: 60 g/L). Figure 3.3.2 reports the time-resolved measurements of the concentration of *A. succinogenes* cells, glucose, and metabolites (succinic acid, acetic acid, formic acid) as well as of pH. All the sugars (glucose, fructose and sucrose) were simultaneously utilized during the fermentation and no diauxic growth was observed. Table 3.3.2 reports the main data regarding fermentation tests carried out with synthetic media containing single sugar (glucose, fructose or sucrose) or with the three sugars mix (SGF medium).

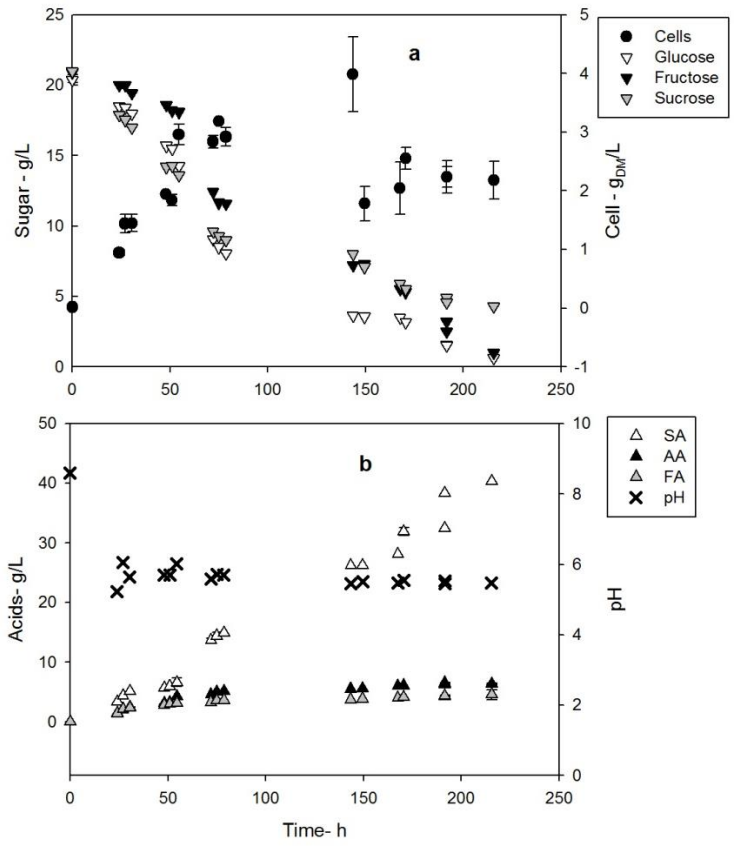

Figure 3.3.2*: Data measured during A. succinogenes fermentation in standard medium supplemented with sucrose, glucose and fructose (SFG); total sugars initial concentration: 60 g/L. a) concentration of biomass and sugars b) pH and concentration of SA, AA and FA.*

A. succinogenes was able to grow and produce succinic acid from all the investigated sugars; glucose was almost completely depleted (ξ_G=0.94), while some sucrose and fructose remained unconverted (ξ=0.70 for both sugars). Sugar-to-succinic acid yield was around 70% for glucose and fructose and slightly lower for sucrose (61%, see Table 3.3.2). Succinic acid production from sucrose was also reported by Jiang et al. (Jiang et al., 2014); they used a fed-batch culture system to prevent sucrose inhibition, obtaining 60.5 g/L of succinic acid with a yield of 82.9%. The sucrose utilization pathways was also investigated in this study, revealing that sucrose is transported and utilized via a sucrose phosphotransferase system, sucrose-6-phosphate hydrolase, and a fructose PTS.

In the mixed sugar test (SGF), SA was continuously produced up to concentration of about 40 g/L and 95% of the total sugar were converted.

Moreover, the succinic acid selectivity The SA selectivity benefits from the co-presence of the four sugars in the fermentation medium benefits from the co-presence of the three sugars in the fermentation medium: it was higher (χ_{SA} =0.84) compared to the single sugar tests (χ_{SA} was about 0.6 for the glucose fructose and sucrose, respectively).

In another investigation by Carvalho et al. (Carvalho et al., 2016) succinic acid production from a feedstock containing glucose, fructose and sucrose was reported; the feedstock investigated was carob pods, an inexpensive by-product of locust bean gum industry. Batch fermentations of *A. succinogenes* with an initial sugar concentration of 30 g/L, resulted in a volumetric productivity of 1.67 g/L*h and a yield of 0.39 g_{SA}/g_S.

High-Sugar-Content Beverages

Table 3.3.3 reports the results of the batch fermentation tests carried out using a HSCBs as a carbon source. For almond syrup cell concentration was not monitored because the turbidity of the medium was too high to allow the measurement.

The tests carried out using raw soft drink (cola and sprite) and the syrup (almond) pointed out that the microorganism did not grow because of the lack of some indispensable nutrients in the fermentation broth. However, it is very interesting to note that when unsupplemented fruit juice were used as fermentation medium, *A. succinogenes* was able to grow and produce succinic acid. The juices, in fact, contain a protein concentration of about 2-4 g/L. The yeast extract commonly used for microorganism cultivation contain 60 to 70 % of protein. Considering that 5 g/L of yeast extract were used for the preparation of the synthetic medium in this study, it means that 3 to 4 g/L of protein were supplemented. Thus, the amount of protein contained in the juice was sufficient to support the microorganism growth.

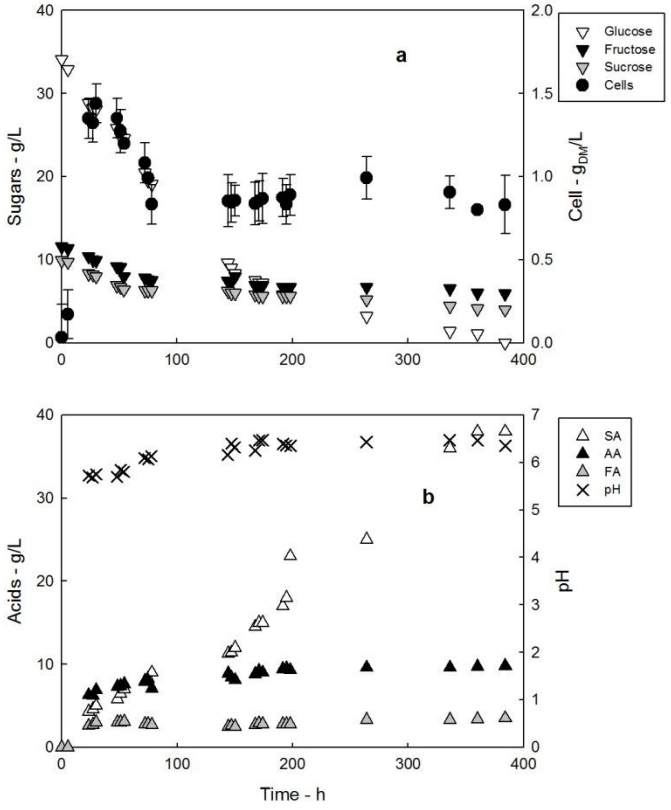

***Figure 3.3.3**: Data measured during A. succinogenes fermentation in supplemented pineapple juice*

Figure 3.3.3 reports the time-resolved profiles of the concentration of *A. succinogenes* cells, sugars, (glucose, fructose, and sucrose), and acids (SA, AA and FA) as well as of pH, measured during a batch culture using pineapple juice as carbon source. After 400h of fermentation, glucose was completely depleted, while some fructose and sucrose remained unconverted. The final concentration of succinic acid is 38 g/L with a yield of 0.83 g/g on total sugars converted. Similar results have been obtained with the unsupplemented pineapple juice in which the amount of succinic acid produced is 37.0 g/L with a better yield of 1.00 g/g.

Table 3.3.3 :*Relevant data of A. succinogenes fermentation tests carried out using HSCB as carbon source.*

		Final SA g/L	ξ_S -	$Y_{AS/S}$ g_{SA}/g_S	χ_{SA} g/g	X_{max} g_{DM}/L	W_{SA}^{max} g/L*h
Pineapple juice	*a*	38.0	0.82	0.83	0.74	1.55	0.18
	b	40.1	0.97	0.75	0.80	3.85	0.43
	c	37.0	0.65	1.00	0.86	1.40	0.22
	d	39.5	0.9	0.75	0.85	3.30	0.24
ACE juice	*a*	28.0	0.57	0.98	0.73	3.40	0.30
	b	30.8	0.75	1.10	0.74	4.00	0.36
	c	24.1	0.48	1.10	0.73	3.70	0.35
	d	29.8	0.74	1.10	0.74	3.30	0.35
Cola	*a*	31.5	0.53	1.00	0.79	1.60	0.20
	b	34.8	0.65	1.07	0.78	0.73	0.34
Almond syrup	*a*	37.3	0.72	1.02	0.80	/	0.21
	b	45.3	0.95	1.10	0.95	1.22	0.35
Sprite	*a*	35.9	0.72	1.08	0.7	3.25	0.23
	b	40.4	1.00	1.11	0.80	3.84	0.34

(a) Supplemented High Sugar Content Beverage; (b) Hydrolyzed Supplemented High Sugar Content Beverage; (c) Unupplemented High Sugar Content Beverage (d) Hydrolyzed Unupplemented High Sugar Content Beverage

Hydrolyzed beverages (HHSCB+) were also tested to verify if the succinic production can be improved when sucrose is hydrolysed to its hexoses component-glucose and fructose. In all the tests, sucrose resulted to be completely hydrolyzed in fructose and glucose.

A significant improvement in the total sugar conversion (about 20%) from HSCB to HHSCB was obtained, as shown in the Figure 3.3.4a, this is explained by the fact that *A.succinogenes* prefers to use glucose rather than sucrose. Succinic acid concentration produced also increased for HHSCB (Figure 3.3.4b).

The analysis of HSCB fermentation suggests that these beverages are a promising resource for succinic acid production, and the process is a potential solution for the remediation of wastewater streams from beverage industries. This is particularly true for the fruit juices, since they do not require to be supplemented with additional protein and vitamin source.

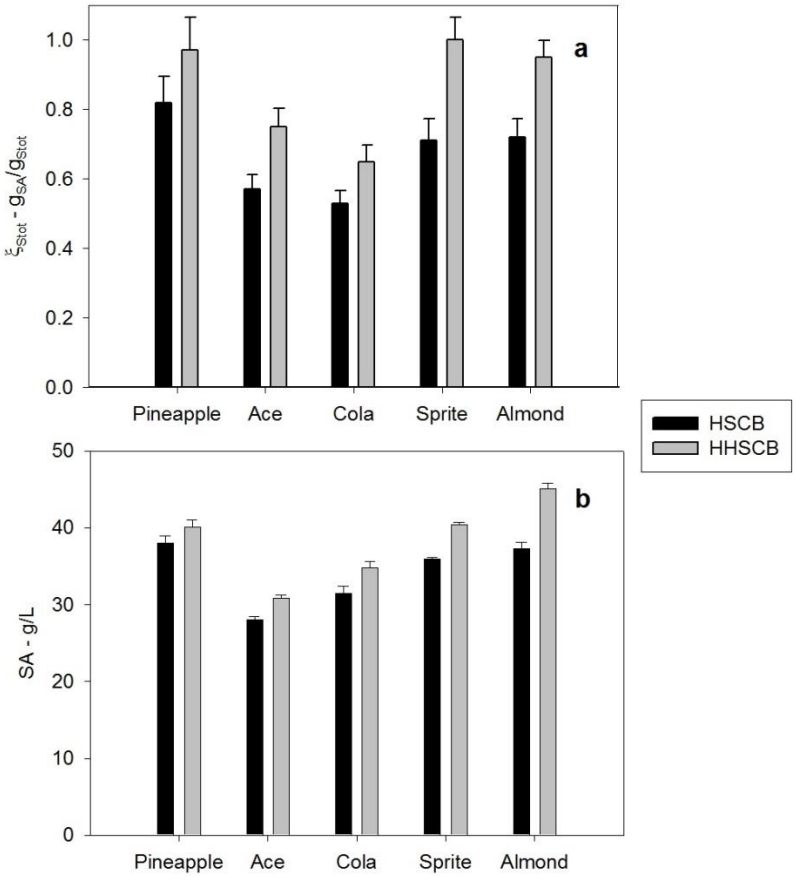

Figure 3.3.4: *HHSCB fermentation tests; a) total sugar conversion degree b) succinic acid production*

Conclusions

The batch fermentations of *A. succinogenes* using high- sugar-content beverages (HSCBs: fruit juices, fruit syrups, soft drinks) and sugar components (glucose, fructose, and sucrose) were successful. *A. succinogenes* grew on HSCBs supplemented with yeast extract and mineral salts. Glucose and fructose conversion was practically complete. Sucrose conversion was very low when mixed with other sugars. HSCBs are a promising substrate for succinic acid production. Considering the huge amount of HSCBs that have to be disposed of in each country, the investigated process has a twofold advantage: to reduce the cost of disposal of these beverages and to produce a high demand bulk chemical, as succinic acid. The conversion degree of the sugar in HSCBs is sufficient to reduce remarkably the disposal cost of this kind of (waste)- water. This reduction coupled with the succinic acid sales suggests that the HSCBs fermentation may be recommended as a process to increase the value of (waste)waters.

Acknowledgments

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4 CONTINUOUS SA PRODUCTION AND INTENSIFICATION OF THE PROCESS

The activity focused on the continuous succinic acid fermentation process characterization. The section 4.1 reports the assessment of the kinetic of growth and succinic acid production by *A. succinogenes* using glucose as carbon source. Tests were carried out in a continuous stirred tank bioreactor (CSTR) operated under controlled pH. Under steady-state conditions, the conversion process was characterized in terms of concentration of glucose, cells, acids, and pH. The section 4.2 reports the continuous succinic acid production by *A. succinogenes* fermentation in a packed bed biofilm reactor (PBBR). The effects of the dilution rate (D) and of different medium compositions (glucose, GX, xylose, GAX) on the PBBR performances were investigated. Moreover, The effect of HMF and furfural - the two main fermentation inhibiting compounds found in lignocellulosic hydrolysates - on succinic acid production was also investigated.

4.1 Kinetics of succinic acid production by *Actinobacillus succinogenes*

Mariateresa Ferone, Francesca Raganati, Giuseppe Olivieri, Piero Salatino, Antonio Marzocchella

Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale – Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

Abstract

The paper reports the characterisation of the growth kinetics of *Actinobacillus succinogenes* DSM 22257 using glucose as carbon source. Tests were carried out in a continuous bioreactor operated under controlled pH. Under steady-state conditions, the conversion process was characterized in terms of concentration of glucose, cells, acids, and pH. The effects of acid - succinic, acetic, and formic - concentration in the medium on the fermentation performance were investigated. The fermentation was interpreted according several models characterized by substrate inhibition and product inhibition. The selected kinetic model of biomass growth and of metabolite production described the microorganism growth rate under a broad interval of operating conditions. Under the investigated operating conditions, results pointed out that: no substrate inhibition was observed; acetic acid did not inhibited the cell growth and the succinic acid production.

Keywords: succinic acid, *Actinobacillus succinogenes*, kinetic, product inhibition, growth model.

Introduction

Succinic acid (SA) is an intermediate in the tricarboxylic acid cycle and one of the end products of mixed-acid anaerobic metabolism (Nghiem et al., 1997). SA has a big potential as a platform chemical for the production of numerous industrial valuable chemical products. A representative spectrum of products are: adipic acid (a precursor for Nylon x,6); 1,4-butanediol (1,4-BDO, a precursor for polyesters and Spandex); tetrahydrofuran (THF, a key solvent and a precursor for poly[tetramethylene ether] glycol); N-methylpyrrolidone (NMP, an key solvent in chemical and lithium-ion battery industries); 2-pyrrolidone (a precursor for pharmaceuticals and vinylpyrrolidone); gamma-butyrolactone (GBL, a precursor for pesticides, herbicides, and pharmaceuticals), and other green solvents and chemicals. Furthermore, the use of SA can be extended to the synthesis of bio-based and/or biodegradable polymers. As a matter of fact, the SA and its derivatives have numerous applications, including surfactants, detergents, biodegradable polymers, foods, fine chemicals, and pharmaceuticals (Zeikus et al., 1999).

The consolidated process to produce SA is via the chemical route from petrol-derived substrates, such as butane or benzene. However, this process is characterized by high costs of the raw materials and environmental drawbacks. In the last decade, the interest in fermentative production of succinic acid has increased because of the possibility of both using inexpensive renewable resources as feedstocks and to propose sustainable production processes. SA can be produced in microbial fermentations by a number of microorganisms, including *Actinobacillus succinogenes* (Guettler et al., 1999), *Anaerobiospirillum succiniciproducens* (Samuelov et al., 1991), *Mannheimia succiniciproducens* (Lee et al., 2002), *Basfia succiniciproducens* (Salvachúa et al., 2016b) and various recombinant strains of *Escherichia coli* (Isar et al., 2006; Jantama et al., 2008). In particular, the *A. succinogenes* has been proved to be a very promising biocatalyst for the industrial application. Indeed, it is able to use a broad spectrum of carbon sources - including several mono-, di-, hexose and pentose sugars (Guettler, 1996).

A. succinogenes produces succinic acid as the main fermentation end-product, along with acetic acid, formic acid, pyruvic acid, and ethanol as minor products (Zeikus et al., 1999). Many investigations have focused on strain improvement, on optimization of the fermentation medium, and on the feeding control to improve succinic acid production (Bretz and Kabasci, 2012; Y. Liu et al., 2008; Zheng et al., 2013). Indeed, the main issue pressing the production of organic acids via fermentation at industrial scale - e.g. citric and lactic acid – is the inhibition behaviour of the produced acid on the cell growth (Nandasana and Kumar, 2008). Therefore, the design and the optimization of fermenter to produce succinic acid requires a deep knowledge of the growth kinetics.

Increased productivity may be achieved by operating fermenters under continuous mode and by increasing the biomass loading by recycling or confining it into the fermenter (Corona-González et al., 2014; Maharaj et al., 2014; Qureshi et al., 2005). Studies on fermentation and simultaneous product recovery to decrease the product inhibition effects have also been carried out (López-garzón et al., 2014).

The design and optimization of bioreactor for the SA fermentation assume a key role for the industrial production. Typically, the production is carried out in batch

fermenters characterized by long dead-time. However, continuous production of SA is likely to outperform batch processes, in particular when high throughput is expected. A complete kinetic characterisation under continuous fermentation conditions should be adopted for a more reliable design/optimization/control of the fermenter. Some studies are present in literature on the kinetic characterization of *A. succinogenes* growth and succinic acid production on various substrate (Lin et al., 2008a; Vlysidis et al., 2011) , but they are typically limited to batch fermentations. Continuous operation allow for proper mass balances through steady-state conditions, which can provide insights into the glucose metabolism of *A. succinogenes*. In a future research work the kinetic model will be used to support the design of a continuous reactors for succinic acid production.

This paper focuses on the growth kinetics of *Actinobacillus succinogenes* cells using glucose as carbon source. The characterization has been carried out operating a CSTR under controlled pH. Fermentation tests were characterized in terms of glucose conversion and acids production. The effects of the acids on the growth rate of *A. succinogenes* cells have been investigated. The study aims at providing kinetic models and parameters for succinic acid production adopting a medium containing glucose.

Materials and Methods

Actinobacillus succinogenes DSM 22257 was supplied by DSMZ. Stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C. The cells were inoculated into 12 mL hungate tubes containing Brain Hearth Infusion Broth (BHI).

The feeding medium consisted of: 5 g/L Yeast Extract (nitrogen source), 1 g/L NaCl, 0.3 g/L Na_2HPO_4, 1.4 g/L NaH_2PO_4,1.5 g/L K_2HPO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.23 g/L $CaCl_2 \cdot 2H_2O$. The medium was sterilized in autoclave (121 °C, 20 min). The carbon source used in the continuous tests was glucose.

Chemicals and yeast extract were from Sigma Aldrich.

Apparatus

The continuous fermentation tests were carried out in the apparatus sketched in Figure 4.1.1. It consists of a fermenter, a pH control unit, a medium feeding system, and a thermostat unit. The fermenter was a 1 L jacketed vessel (Pyrex®) with a working volume of 0.4 L. The pH control unit consisted of a pH probe connected to a pH controller, a peristaltic pump (Gilson Minipuls 3) for the delivery of a 10 M NaOH solution. The fermentation broth was fed by means of a peristaltic pump. A gas stream was spread at the bottom of the fermenter to mix the culture and to provide CO_2. The gas stream was sterilized by filtration (cut-off 0.2 μm, Millipore). The reactor was heated at the set temperature by means of a hot water stream delivered by a thermostatic water bath and circulating through the reactor jacket. The liquid phase volume in the reactor was adjusted by fixing the level of the overflow duct.

Mixing was also provided by means of magnetic agitation. The reactor vessel was sterilized in autoclave.

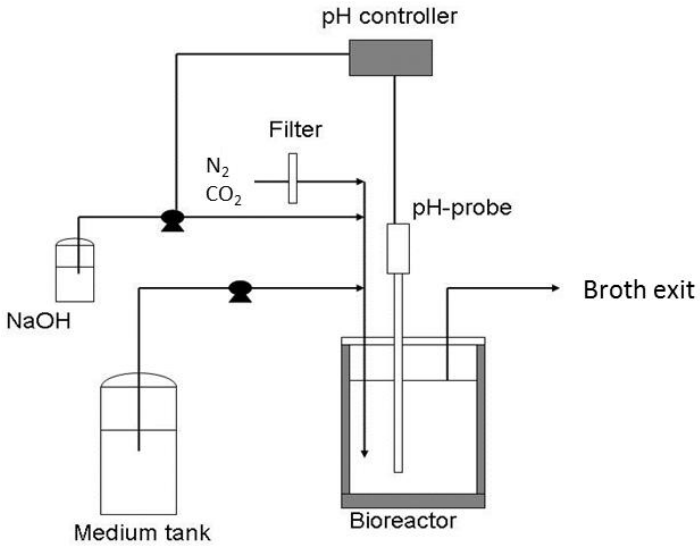

Figure 4.1.1: *Outline of the apparatus adopted for continuous tests.*

Analytical methods

Cell density was measured as optical absorbance at 660 nm (OD_{660}) using a spectrophotometer (Cary- 50 Varian). Dry cell weight (DCW) was measured by harvesting the cell present in 10-mL samples by centrifugation at 5000 rpm for 15 min at 4°C. Cell pellets were rinsed twice with distilled water and recovered by centrifugation, then dried at 60 °C for 24 h under vacuum. The processing of dry cell weight concentration and optical absorbance measurements provided that 1 OD_{660} was equal to 0.38 g_{DM}/L.

The concentration of soluble species was measured in the liquid phase after spinning down the cells by centrifugation (13000g, 10min). Sugar and organic acid concentrations were measured by means of a high performance liquid chromatography (HPLC) (HP1250 working station system - Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9 µm; Bio-Rad Chemical Division, Richmond, CA). Analytes were detected by UV absorbance (Agilent Technologies, G1315D) and refractive index (Agilent Technologies, G1362A). H_2SO_4 5mM was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20 µL.

Operating conditions and procedures

300 µL of stock culture were transferred into four 12-mL Hungate tubes containing the pre-culture medium (BHI broth). The pre-cultures were incubated for 24 hours, then 40 mL of active culture were inoculated in the reactor containing 0.4 L of fermentation medium. Typically, after 18-24 h of batch culture the glucose-bearing stream was fed to the reactor at the selected volumetric stream rate. The liquid flow rate was selected to provide the pre-set dilution rate (D=Q/V). The dilution rate was tuned between 0.03 and 0.65 h^{-1}. The culture was periodically sampled to measure biomass, sugar and metabolite concentrations until steady-state conditions were reached 4–5 times the liquid space–time were attended.

The pH was set at 6.80. The reactor temperature was set at 37°C. The gas stream fed to the fermenter was a mixture of 80% N_2 and 20% CO_2 and the feeding rate was 1.5 vvm CO_2. The fermenter working volume was set at 0.4 L. The glucose concentration in the fermenter feeding was set at 60 g/L and 150 g/L.

Theoretical framework

Growth rate model

Previous investigations (Ferone et al., 2017b; Lin et al., 2008a) carried out under batch conditions have pointed out that the growth kinetics of *A. succinogenes* is characterized by inhibition by glucose and by the produced acids. According to the reported observations, growth kinetics models characterized by substrate inhibition were taken into account:

Haldane-type inhibition: $\mu = \mu_{max} \frac{S}{S+K_S+{S^2}/{K_i}}$ (4.1.1)

Luong-type inhibition: $\mu = \mu_{max} \frac{S}{S+K_S}\left(1-\frac{S}{S_{max}}\right)^{n_S}$ (4.1.2)

where μ is the specific growth rate, μ_{max}, K_S, K_i, $n_S$and S_{max} constant of the models. The Haldane-type model is characterized by finite, though small cell growth rate even at very large substrate concentrations (S→∞). The Luong-type model is characterized by full inhibition as the concentration of substrate approaches a critical value (S_{max}).

Models typically used to describe product inhibition kinetics are the followings:

Luong-type inhibition $\mu = \mu_{max}\left(1-\frac{P}{P_{max}}\right)^{n_P}$ (4.1.3)

Ierusalimsky-type inhibition $\mu = \mu_{max}\frac{1}{1+{P}/{K_P}}$ (4.1.4)

Aiba-type inhibition $\mu = \mu_{max}e^{-P/K_P}$ (4.1.5)

Equation (4.1.3) is characterized by fully inhibition as the concentration of inhibitor metabolites approaches a critical value (P_{max}). Equations (4.1.4) and (4.1.5) are characterized by gradual inhibition as the inhibitor metabolite concentration increases.

In the present investigation the combinations of the reported models were considered to provide the optimal multiproduct/substrate-inhibited model to describe the specific growth of *A. succinogenes*.

The cell growth rate was measured by processing data measured during steady-state conditions in a chemostat. Steady- state was assumed when the absolute deviation of metabolites concentration, captured over a period of at least 12 h, did not exceed 10 % of the mean value. Indeed, results of the continuous tests were worked out considering the mass balance on cells extended to the continuous reactor. Under steady state conditions the biomass balance reads:

$D = \mu$ (4.1.6)

Design of experiments

The parameters of the proposed models were assessed according the multistep design of experiments described hereinafter.

Step (1) – A first test campaign was carried out feeding continuously the fermenter with a 60 g/L glucose medium: the continuous steady-state tests were characterized at dilution rate D set between 0.03 and 0.6 h^{-1}. A second test campaign was carried out feeding continuously the fermenter with a 150 g/L glucose medium: the continuous steady-state tests were characterized at dilution rate D set between 0.22 and 0.58 h^{-1}. No metabolites were supplemented to the feeding in both test campaigns. Under these operating conditions, succinic acid was produced at concentration up to about 40 g/L, and the acetic acid and formic acid concentration were negligible. Therefore, the assessment of the role of glucose and SA on the cell growth was carried out without any interference from AA and FA.

Step (2) – A test campaign was carried out supplementing the fermenter feeding with AA and FA. In particular, the concentration of acetic and formic acid was forced at values higher than that coupled with the glucose fermentation. The processing of data measured during this campaign were addressed to assess the inhibitory effect of acetic and formic acid on the cell specific growth rate.

Model parameters estimation

Preliminary estimate of μ_{max}, K_{Glu} and of the inhibition constants. Data processing of the measurements carried out during the Step (1) continuous fermentation tests were addressed to the preliminary assessment of the parameters of the cell growth models. In particular, the role of the glucose and of the SA on the cell growth was at the hand. The full set of models investigated is reported in Table 4.1.1. The kinetic parameters (μ_{max} , K_{Glu} and the inhibition constant) were assessed according to the best-fit procedure applied to data measured during Step (1) tests. Under these operating conditions, the SA was the only acid that may contribute to the inhibition.

Exact determination of μ_{max}, K_G, and of the inhibition constants. Data processing of the measurements carried out during the Step (2) continuous fermentation tests were addressed to assess all the parameters of the cell growth models. In particular, the role of the glucose, SA, AA and FA on the cell growth was at the hand. The full set of models investigated is still reported in Table 4.1.1. The complete set of the model parameters was assessed by a parametric inference procedure applied to the model by the regression of experimental data measured during steady-state fermentations carried out under controlled supplement of acids in the feeding (Step 2). The preliminary estimation of the kinetic parameters from the previous step were used as the starting point of the multivariate best-fit convergence procedure in the second step.

The parameters of the proposed model were estimated using nonlinear, weighted, least squares method by minimizing the sum of squared errors between the estimated and experimental values. Simulated annealing, a stochastic optimization algorithm implemented in MATLAB was used. The soundness of the model was tested through the assessment of the average squared correlation coefficients (R^2) and of the mean square deviation (δ^2) between the simulation results and the experimental data.

***Table 4.1.1**: Equation used in step 1 to fit the experimental data and parameters estimated from each model*.

	Model description	**Equation**
Step 1		
7	Glucose: Luong-type inhibition; SA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \left(1 - \frac{G}{G_{max}}\right)^{n_G} \left(1 - \frac{SA}{SA_{max}}\right)^{n_{SA}}$
8	Glucose: Haldane-type inhibition; SA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G + {G^2}/{K_i}} \left(1 - \frac{SA}{SA_{max}}\right)^{n_{SA}}$
9	Glucose: Monod; SA: Ierusalimsky-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i}$
10	Glucose: Monod; SA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \left(1 - \frac{SA}{SA_{max}}\right)^{n_{SA}}$
Step 2		
11	Glucose: Monod; SA, AA and FA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \left(1 - \frac{SA}{SA_{max}}\right)^{n_{SA}} \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \left(1 - \frac{FA}{FA_{max}}\right)^{n_{FA}}$
12	Glucose: Monod; SA, AA and FA: Ierusalimsky-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i} \frac{K_{AA}^i}{AA + K_{AA}^i} \frac{K_{FA}^i}{FA + K_{FA}^i}$
13	Glucose: Monod; SA and FA: Ierusalimsky-type inhibition; AA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i} \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \frac{K_{FA}^i}{FA + K_{FA}^i}$
14	Glucose: Monod; SA and FA: Ierusalimsky-type inhibition; AA: Aiba-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i} e^{-AA/K_{AA}} \frac{K_{FA}^i}{FA + K_{FA}^i}$
15	Glucose: Monod; SA: Ierusalimsky-type inhibition; AA and FA: Luong-type inhibition	$\mu = \mu_{max} \frac{G}{G + K_G} \frac{K_{SA}^i}{SA + K_{SA}^i} \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \left(1 - \frac{FA}{FA_{max}}\right)^{n_{FA}}$

Assessment of energetic yield

According to Bauchoop and Elsden (1960) [22], the ATP yield (Y_{ATP}) is expressed as the amount of dry weight of the produced microbial cells per gmol of ATP. The interpretation and the assessment of Y_{ATP} was thoroughly studied in the past and the main results were:

- the maximum value of Y_{ATP} ranges between 28.6 and 32.1 g_{DM}/mol_{ATP} for microorganisms growing on glucose (Meyer and Papoutsakis, 1989);
- the Y_{ATP} of a given microorganism may change with the specific growth rate as well as with the media composition. In particular, Pirt (Pirt, 1965) proposed the Eq. (4.1.16) for the relationship between Y_{ATP} and the specific growth rate:

$$\frac{1}{Y_{ATP}} = \frac{1}{Y_{ATP}^{max}} + \frac{m}{\mu} \quad (4.1.16)$$

where m is the maintenance requirement coefficient.

- the maintenance coefficient depends on the specific growth rate (Pirt, 1982):

$$m = m_0 + m_1\left(1 - \frac{\mu}{\mu_{max}}\right) \quad (4.1.17)$$

where μ_{max} is the maximum specific growth rate, m_0 and m_1 the maintenance coefficient at $\mu = \mu_{max}$ and $\mu = 0$. Assuming m_0 negligible, the combination of Eq.s (4.1.16) and (4.1.17)

yields:

$$Y_{ATP} = Y_{ATP}^{max}\frac{\mu}{\mu + Y_{ATP}^{max} \times m_1\left(1-\left({}^{\mu}/_{\mu_{max}}\right)\right)} \quad (4.1.18)$$

The ATP produced during both glycolysis and acids production is diverted for growth and maintenance of cells. Accordingly, the biomass yield (Y_{ATP}) may be experimentally assessed taking into account the biomass and metabolites produced, provided that the stoichiometric relationship between metabolites and mole of ATP produced is known. The glucose conversion path during the *A. succinogenes* fermentation under continuous conditions may be lumped into a single reaction related to the formation of SA, since AA and FA formation is negligible (4.1.19):

$$C_6H_{12}O_6 + 2\,CO_2 + 2\,NADH + 2\,H^+ \rightarrow 2\,SA + 2\,NAD^+ + 2\,ATP + 2\,H_2O \quad (4.1.19)$$

Equation (19) suggests that the mass of acid produced per mole of ATP produced ($Y_{SA/ATP}$) is 118 g_{SA}/mol_{ATP}. According to the definition, the ATP yield may be calculated as:

$$Y_{ATP} = Y_{SA/ATP} \times Y_{X/SA} = 118\,Y_{X/SA} \quad (4.1.20)$$

where $Y_{X/SA}$ may be assessed as:

$$Y_{X/SA} = \frac{r_X}{r_{SA}} = \frac{DX}{D(SA - SA_0)} = \frac{X}{(SA - SA_0)} \quad (4.1.21)$$

Results and Discussion

Figure 4.1.2 reports the concentration of biomass, glucose and succinic acid measured during continuous fermentations carried out in the apparatus showed in Figure

4.1. 1. The feeding stream contained 60 g/L glucose without any acid supplement. The concentration data reported in Figure 4.1.2 were measured under steady-state conditions were established. The dilution rate was set between 0.03 and 0.60 h^{-1}. At low D, the glucose conversion was quite complete, the SA approached the maximum concentration and the cell concentration was not very high. As D was increased, the glucose conversion and the SA concentration decreased. The plot of the biomass concentration vs. D is characterized by a maximum of 1.73 g_{DM}/L of cells at D=0.2 h^{-1}. The analysis of the plot of substrate/SA concentration vs. dilution rate suggests a process characterized by product inhibition or combined substrate/product inhibition. Indeed, the specific cell growth rate decreases with increasing SA concentration in the effluent.

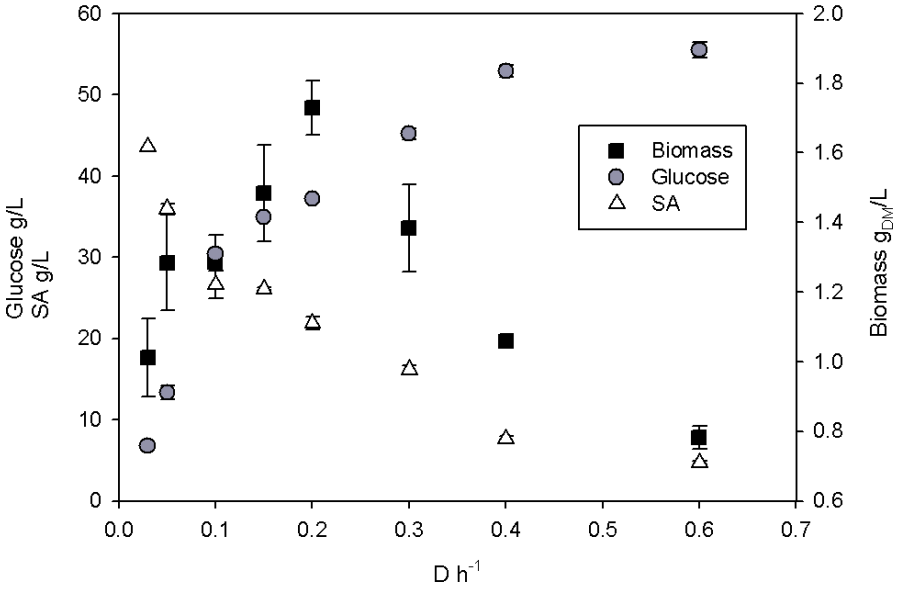

Figure 4.1.2*: Biomass, glucose and SA concentration vs. dilution rate measured during continuous fermentation under steady state conditions. Feeding: 60 g/L glucose; acids were not supplemented.*

The SA/AA ratios ranged between 11.5 and 39.6 g/g for glucose feed concentrations of 60 g/L. It has been shown that a relative increase in the flux toward the oxidative pentose phosphate pathway can provide additional reduction power that enables increased flux through the reductive branch of the TCA cycle (Bradfield and Nicol, 2016), leading to increased SA yields and reduced by-product formation. Moreover a single membrane-bound hydrogenase (Asuc_1277-83) was also identified *in A. succinogenes* genome. Hydrogenases catalyze the reversible reduction of protons to hydrogen gas. They are found in a wide variety of microorganisms and enable them to use H_2 as a source of reductant under either aerobic or anaerobic conditions (Adams, 1990).

It should be mentioned that biofilm formation was unavoidable when the reactor was operated for more than 10 days. Therefore, fermentations were stopped when biofilm formation occurred to prevent erroneous data interpretation. The natural capability of *A. succinogenes* to form biofilm was proved by Urbance et al. (2004) (Urbance et al., 2004) and all the continuous studies on this strain resulted in biofilm formation under prolonged operation (Bradfield and Nicol, 2014; van Heerden and Nicol, 2013).

Effect of glucose on growth

Previous investigations carried out in batch conditions (M. Ferone et al., 2017b; Lin et al., 2008a) pointed out that the cell growth is inhibited by high concentration of glucose. Moreover Urbance et al., (Urbance et al., 2004) proved that in batch fermentation, *A. succinogenes* can tolerate initial glucose concentrations up to of 160 g/L. According to the previous indications, a test campaign was carried out by feeding the fermenter with a continuous stream bearing glucose at 150 g/L.

Data regarding the test campaigns feeding the fermenter with a continuous stream bearing glucose at 60 and at 150 g/L are reported in Table 4.1.2. Under the selected operating conditions, the concentration of acetic and formic acids produced was lower than the inhibition concentration previously reported in the literature (16 and 44 g/L respectively, for formic and acetic acid (Lin et al., 2008a)). According to these observation, it was assumed that: i) the inhibition effect of the FA and AA may be neglected; ii) the SA inhibition was active.

Table 4.1.2*: Main results of the continuous tests carried out feeding the chemostat with a medium containing glucose at concentration G_0.*

G_0 (g/L)	D=μ (h^{-1})	Concentration in the reactor (g/L)			
		Glucose	SA	AA	FA
60	0.03	6.8	43.6	1.1	0
60	0.05	13.3	35.9	0.8	0.9
60	0.1	30.4	26.6	0.7	0.3
60	0.15	34.9	26.1	0.4	0.3
60	0.2	37.1	21.8	0.8	0.7
60	0.3	45.2	16.1	0.6	0.3
60	0.4	52.9	7.6	0.6	0.3
60	0.6	55.5	4.6	0.4	0
150	0.22	99.5	18.7	0	3.3
150	0.37	107.2	9.7	0	0
150	0.47	118.0	7.9	0	0
150	0.58	130.0	5.0	0	0

According to the Step 1 procedure described in the "§ 3.2 and § 3.3", data measured during the fermentation tests carried out at glucose concentration in the chemostat feeding of 60 g/L and 150 g/L were processed. The results of the fitting with Eq. (4.1.7) and (4.1.8) from Table 4.1.1 suggest that no glucose inhibition occurred in under the operating conditions tested because the inhibition constants were quite high for all the investigated models (See Table 4.1.3). For this reason, the effect of glucose in the cell growth kinetics was modelled according to the classical Monod model. Data reported in Table 4.1.2 were worked out according to Eq. (4.1.9) and Eq. (4.1.10) for a rough estimate of μ_{max}, K_G and the inhibition constant for succinic acid:

Estimated parameters for Eq. (4.1.9):

μ_{max}= 1.55 h^{-1}

K_G= 9.60 g/L

K_{SA}= 3.64 g/L

Estimated parameters for Eq. (4.1.10):

μ_{max}= 0.79 h^{-1}

K_G= 0.69 g/L

SA_{max}= 132 g/L

n_{SA} = 8

***Table 4.1.3**: Estimated parameters from the Step 1 procedure*

Model	**Estimated parameters**								**Accuracy parameters**	
Step 1										
	μ_{max} h^{-1}	K_G g/L	K_i g/L	G_{max} g/L	n_G -	K_{SA} g/L	SA_{max} g/L	n_{SA} -	R^2 -	δ^2 -
7	0.82	3.24	-	300	0.1	-	150	9.13	0.97	0.012
8	1	12	500	-	-	-	150	8.87	0.97	0.012
9	1.55	9.6	-	-	-	3.64	-	-	0.98	0.01
10	0.76	0.69	-	-	-	-	132	8	0.97	0.011

Effect of acids on growth

According to previous studies, *A. succinogenes* growth is affected by inhibition caused by the main end product of the fermentation (SA, AA and FA), both under batch (Lin et al., 2008a) and continuous conditions (Brink and Nicol, 2014).

Table 4.1.4 reports data resulting from tests carried out with the aim of determining the critical concentrations of SA, AA and FA. The feed was supplemented with either acetic or formic acid. According to the Step 2 procedure described in "§ 3.2 and § 3.3", the exact estimation of the parameters in Equations from (4.1.11) to (4.1.14) was accomplished by the regression of data in Tables 4.1.1 and 4.1.2. The multivariate best-fit procedure with Eq. (4.1.14) provided:

μ_{max}= 1.43 h^{-1}

K_G= 6.96 g/L

K_{SA}= 4.10 g/L

AA_{max}= 80 g/L

n_{AA}= 0.1

FA_{max}= 45.6 g/L

n_{FA}= 0.53

μ_{max} and K_G assessed in the Step 2 are only slightly different from the values obtained from Step 1. The inhibition constant for succinic acid (K_S), indicates gradual

inhibition as the acid concentration increases. AA_{max} and FA_{max} rapresent, respectively, the critical concentrations at which cell growth is completely ihnibited; n_{AA} and n_{FA} denote the degree of product inhibition (dimensionless).

The assessed data are clearly valid within the operating conditions set. However, the wide intervals of dilution rate, glucose and metabolites concentration provides a sound spectrum of the model application.

The inhibitory effects of organic acids on bacterial growth is known to depend on the pH of the fermentation medium, the dissociation constants of acids (pKa), and their concentrations (Palmqvist and Hahn-Hägerdal, 2000). Moreover, the inhibitory effect of weak acids on microbial growth is also related to their diffusion into the cytosol where they cause the decrease of the intracellular pH. In these study, acetic acid contributed just barely to the growth inhibition, considering the very low value for n_{AA}. Critical concentrations of FA reported in the literature for the same bacterium are 10.8 g/L (Lin et al., 2008a) and 35.2 g/L (Li et al., 2010a). Regarding the AA, the reported critical inhibitory concentrations are 33.7 g/L (Lin et al., 2008a) and 20 g/L (Li et al., 2010a).

Table 4.1.4*: Main results of the continuous tests carried out feeding 60 g/L glucose medium supplemented with either acetic acid or formic acid.*

Supplemented product	**D=μ (h^{-1})**	**Concentration in the effluent stream (g/L)**			
		Glucose	SA	AA	FA
Acetic acid (AA)	0.045	29.7	28.9	20.1	0
	0.25	38.9	20.6	18.9	0
	0.31	40.3	15.9	20.2	0
	0.49	48.9	8.4	20.2	0
Formic acid (FA)	0.23	41.5	16.1	0	6.7
	0.35	52.6	9.6	0	6.9
	0.50	56.5	5.3	0	6.9
	0.65	58.9	3.6	0	6.7

Considerations on growth kinetics

Figure 4.1.3 reports a parity plot of the theoretical value of the specific growth rate – Eq. (4.1.14) combined with model parameters reported in Table 4.1.4 – vs. the experimental value of the specific growth rate. It may be observed that the error between the expected value and the experimental value is less than 10%. This observation suggests that the proposed kinetics Eq. (4.1.14) and the multistep parameter inference procedure used were reliable and the growth kinetics of *A. succinogenes* on glucose has been correctly assessed.

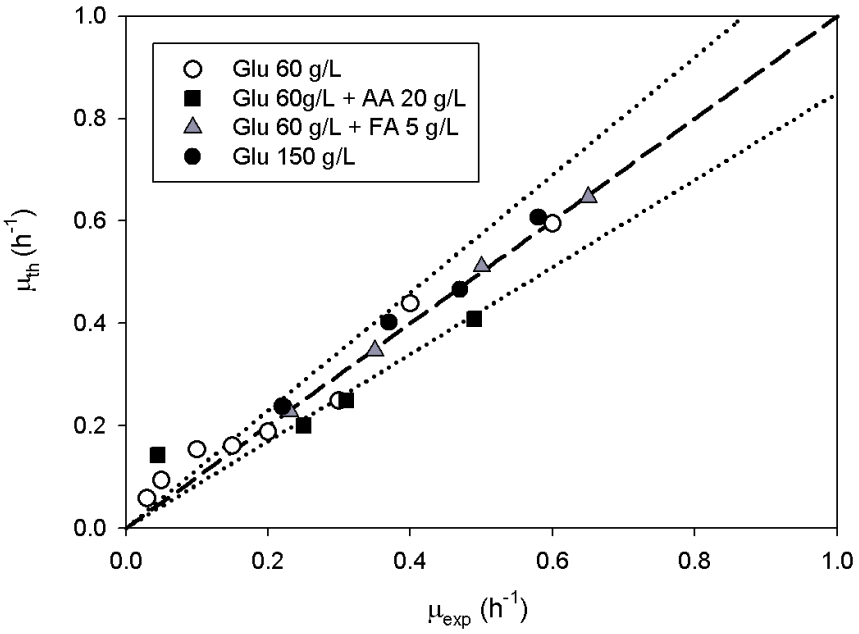

Figure 4.1.3: *Theoretical (Eq. (4.1.14) vs. experimental specific growth rate of A. succinogenes. Dotted lines: ±10% error.*

Table 4.1.5 lists the main kinetic parameters of the *A. succinogenes* growth assessed in the present work and reported in the literature. The main differences are as follows.

- Under continuous operating mode, *A. succinogenes* growth is not affected by high glucose concentration.
- The saturation constant for glucose assessed under continuous mode of operation is larger than that assessed under batch conditions (see Table 4.1.6).
- The maximum specific growth rate (μ_{max}) depends remarkably on the operating condition mode. In particular, the μ_{max} assessed under continuous fermentation is about three-fold that assessed under batch conditions.
- The SA inhibition constant of 4.10 g/L confirms the strong inhibition caused by the acid.
- Under continuous operating mode, *A. succinogenes* could tolerate higher acid concentration compared to that reported in literature for batch mode.

The model gave satisfactory results: the squared correlation coefficient (r^2) were higher than 0.95 and of the mean square deviation (δ^2) were below 0.04 (Table 4.1.5).

***Table 4.1.5**: Kinetic parameters assessed according to the proposed model (see Table 4.1.1) and from the literature.*

Sugar	μ_{max}	K_S	K_i	C_S^{max}	n_S	K_i^{SA}	SA_{max}	n_{SA}	AA_{max}	n_{AA}	FA_{max}	n_{FA}	Operation mode	Reference
Glucose	1.43	6.96				4.10			80.0	0.1	45.6	0.53	Continuous	This work
Glucose	0.39	0.1	15.5										Batch	(M. Ferone et al., 2017b)
Xylose	0.10	4.82											Batch	(M. Ferone et al., 2017b)
Mannose	0.17	9.49											Batch	(M. Ferone et al., 2017b)
Arabinose	0.11	7.14											Batch	(M. Ferone et al., 2017b)
Glucose	0.50	2.03		155	0.6		104.2	1	44.2	1	16.0	1	Batch	(Lin et al., 2008a)
Xylose*	0.39	0.70	55.5				55.0	2.3	38.0	2.3	18.0	2.3	Batch	(Pateraki et al., 2016)
Glycerol	0.12	2.90	15.4				45.6	1.07					Batch	(Vlysidis et al., 2011)

*****Kinetic parameters referred to the mixed sugars medium, where xylose is the most abundant one.

Energetic yields

The data measured during the fermentation tests reported in Figure 4.1.2 and Tables 4.1.2 and 4.1.4 were worked out in agreement with Eq.s (4.1.20) and (4.1.21) to assess the values of Y_{ATP} and m_1. The best fitting procedure applied to Y_{ATP} data according Eq. (4.1.18) and setting $\mu_{max} = 1.43\ h^{-1}$ yielded:

$$Y_{ATP}^{max} = 23.9\ g_{DM}/mol_{ATP} \qquad m_1 = 0.016\ mol_{ATP}/g_{DM}h$$

The satisfactory agreement between the plot of Eq. (4.1.18) in Figure 4.1.4.and the experimental data supports the validity of the proposed model.

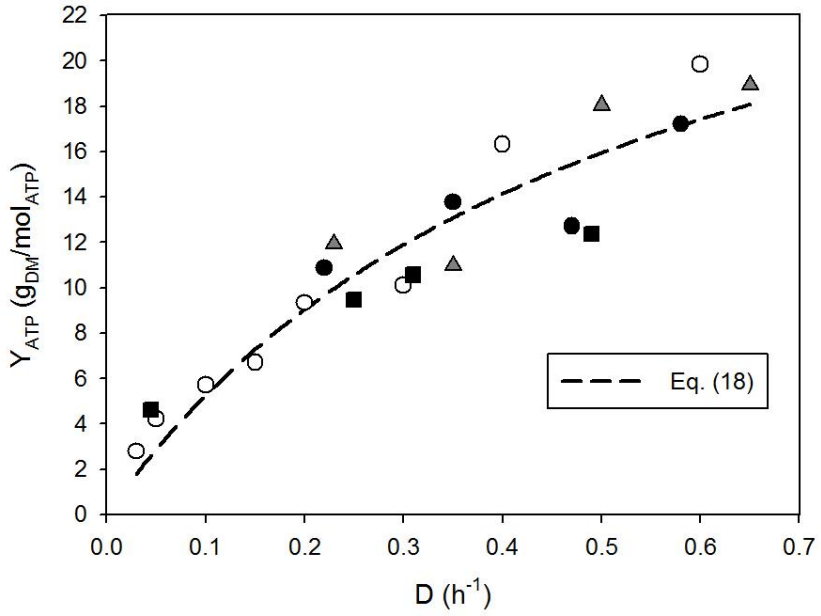

***Figure 4.1.4** Y_{ATP} vs specific growth rate. Symbols are reported in Fig. 4.1.3.*

Conclusion

The kinetics of *A. succinogenes* growth on glucose was investigated, assessing the influence of glucose and succinic, acetic and formic acids on growth. The experimental results were successfully correlated by a multiple product-inhibited, interacted growth model. Experimental results pointed out the individual inhibitory effect of succinic, acetic and formic acid. The kinetic model is relevant to design and optimize a continuous bioreactor to produce succinic acid, and to characterize the dynamic behavior of bioreactors.

The microorganism maintenance requirement was also investigated. The ATP yield was assessed according to Pirt model. The agreement between the expected and experimental yields was satisfactory.

Acknowledgements

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Nomenclature

S_{max}, P_{max} ,	Critical concentrations of substrate or products (g/L)
AA, FA, G, SA,	Concentration of acetic, formic and succinic acid, glucose (g/L)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
D	Dilution rate (h^{-1})
K_S	Substrate saturation constant (g/L)
K_i	Substrate inhibition coefficient (g/L)
K_P	Inhibition coefficient for products (g/L)
n_i	Exponent of inhibitory products
R^2	Coefficient of determination
Y_{ATP}	ATP yield (g_{DM}/mol_{ATP})
Y_{ATP}^{MAX}	maximum ATP yield (g_{DM}/mol_{ATP})
$Y_{X/SA}$	mass ratio between biomass and succinic acid (g_{DM}/g)
$Y_{SA/ATP}$	ratio between succinic acid and ATP moles (g/mol_{ATP})
X	cell concentration (g_{DM}/L)
δ^2	Mean square deviation

4.2 Continuous succinic acid fermentation by *Actinobacillus succinogenes* in a packed bed biofim reactor

Mariateresa Ferone, Francesca Raganati, Alessia Ercole, Giuseppe Olivieri, Piero Salatino, Antonio Marzocchella

Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

Abstract

Continuous succinic acid production by *Actinobacillus succinogenes* fermentation in a packed bed reactor (PBR) was investigated. The PBR was a 5 cm ID, 8 cm high glass tube with a 4.5 cm bed of 3mm Tygon rings, as carriers. The reactor was operated at dilution rate set between 0.64 h^{-1} and 2.4 h^{-1} and it was feed with different media that simulate the composition of a lignocellulosic hydrolysate. The effects of the operating conditions tested, dilution rate (D) and of different medium compositions (glucose, GX, xylose, GAX media) on the PBR performances were investigated. The highest SA productivity of 35.0 g/L*h and the maximum SA concentration was achieved at a D= 1.9 h^{-1}.

The effect of HMF and furfural on succinic acid production was also investigated. HMF resulted to reduce succinic acid production by 22.6%, while furfural caused a reduction of 16% in SA production at the same dilution rate.

Keywords: biorefinery, succinic acid, *Actinobacillus succinogenes*, biofilm, lignocelluloses.

Introduction

Sustainable production of chemicals and fuels from renewable resources is a priority for the modern societies. Indeed, the growing awareness of the environmental impact of petrochemical processes has increased the interest for alternative routes for a sustainable productions of commodities. According to this scenario, biorefineries offer an excellent opportunity to replace the oil-refinery with the bio-based derived products (Cherubini, 2010).

Organic acids - in particular bicarboxylic acids - are expected to play a key role in the feasibility of future biorefineries because their huge potential as platform molecules. Succinic acid (SA) - a four carbon dicarboxylic acid produced as an intermediate in the tricarboxylic acid (TCA) cycle - is a very interesting bicarboxylic acid that can be produced by fermentation of renewable resources. The high potential of the SA has been pointed out by the US Department of Energy that included it among the 12 top value-added chemical produced from biomass (Werpy and Petersen, 2004). Indeed, the SA is currently used in the food industry, as a pH regulator and as a flavoring agent, in the pharmaceutical industry, as additive for the preparation of drugs, in the agricultural food and as ion chelator and surfactants (Zeikus et al., 1999). . Because of its structure, SA can be also used as a building block chemical and converted to 1,4-butanediol, γ-butyrolactone, tetrahydrofuran, N-methyl-2-pyrrolidone, 2-pyrrolidone, succinimide, succinic esters, maleic acid/maleic anhydride (Sauer et al., 2008). The industrial success of the SA produced via the biotechnological route depends on the production cost. The current price of succinic acid produced via the petrolchemical route is about 2.94 $/kg (Taylor et al., 2015) and any efforts should be addressed to reduce the production cost around 1$ /kg to propose the bio-SA as a potential alternative to the chemical route, as it is required for the production of commodity products by the chemical industry (Hermann et al., 2007). Several companies - such as BioAmber, Myriant, Succinity, and Reverdia - have developed processes for the production of bio-succinic acid by proprietary organisms and strain. However, the current commercial production of SA is based on the use of pure sugars derived from starch- based raw materials that potentially competes with food resources (Jansen and van Gulik, 2014).

The key issues for the success of industrial processes for the production of succinic acid via the biotechnological route include the selection/development of a SA producing microorganism, the selection of the feedstock ,the specific productivity of the fermenters and the development of an efficient recovery process for SA.

A potential microbial platform to produce SA is *Actinobacillus succinogenes*: a microorganism characterized by the best bench-scale performances (Guettler, 1996; Yan et al., 2013; Zheng et al., 2013). It has been pointed out that this bacteria can produce SA at high yields and concentration during mixed-acid, batch fermentation, by using a variety of carbon source (Jiang et al., 2014; Liang et al., 2013; Wan et al., 2008). However, according to previous studies (M. Ferone et al., 2017b; Lin et al., 2008a), *A. succinogenes* growth is inhibited by the acids produced during the fermentation and this feature reduces the volumetric productivity of batch processes and increases the dead- times. These drawbacks can be addressed by the use of single culture biofilm reactors; the main advantages of this typology of reactors include high cell density achieved, operability at high dilution rate without cell washout, high specific productivity, and the possibility to reuse the biofilm support (Qureshi et al., 2005). Moreover, biofilms are known for their stability to long-term continuous operation and the enhanced tolerance to toxic compounds (Gross et al., 2007; Raganati et al., 2016).

In addition to the choice of the biocatalyst and of the reactor type, the feedstock selection plays a key role in the economics of the process (Tan et al., 2014). Lignocellulosic biomass are generally considered an ideal feedstock for the production of bio-products because of their low cost, high availability and un-competitiveness with food sources (Anwar et al., 2014; Saini et al., 2014). Pretreatment of lignocellulosic biomass should be carried out via combined thermo-chemical and enzymatic treatment in order to produce C5 and C6 sugars; however, microbial inhibitors - such as furfural, hydroxymethylfurfural (HMF), acetic acid, and low molecular weight phenolic compounds – may be produced during the pretreatment process and they reduce the performance of the SA production process.

The present contribution regards the continuous production of succinic acid by wild-type biofilm of *Actinobacillus succinogenes* in a packed bed reactor. The performance of the reactor was assessed under a wide range of dilution rate and by feeding the reactor with streams bearing several sugars (single and mixed). The continuous fermentation process was characterized in terms of succinic acid concentration, productivity and selectivity as well as sugar conversion. In addition, the effect of two putative fermentation inhibitors, such as furfural and HMF, was investigated.

Methods

Microorganism and media

Actinobacillus succinogenes DSM 22257 was supplied by DSMZ. Stock cultures were reactivated according to the supplier procedure. Reactivated cultures were stored at -80 °C. The thawed cells were inoculated in 15 mL Hungate tubes containing 12 mL of containing Brain Hearth Infusion broth (BHI). Cells were grown under anaerobic conditions for 24 h at 37 °C. Then the pre-cultures were transferred into fermentation bottles.

The feeding medium consisted of: 5 g/L Yeast Extract (nitrogen source), 1 g/L NaCl, 0.3 g/L Na_2HPO_4, 1.4 g/L NaH_2PO_4,1.5 g/L K_2HPO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.23 g/L $CaCl_2 \cdot 2H_2O$. The medium was sterilized in autoclave (121 °C, 20 min). The carbon source used in the continuous tests was glucose.

Chemicals and yeast extract were from Sigma Aldrich.

Apparatus

The apparatus used for the continuous fermentation consisted of a fixed bed reactor, liquid pumps, a heating apparatus, a system for pH control and on-line diagnostics (Figure 4.2. 1). The fixed bed was at the bottom of a 166-mL glass lined bottle (5 cm ID, 8.5 cm high) jacketed for the heat exchange. The liquid phase volume in the reactor (the reaction volume) was set by changing the level of the overflow duct. Carbon dioxide was sparged at the reactor bottom to support anaerobic conditions and to provide the CO_2 for the succinic acid production pathway. The system for pH control consisted of a pH-meter, a peristaltic pump, a vessel with NaOH 0.3 M solution, and a pH controller.

The reactor with the carriers was sterilized in autoclave at 121 °C for 20 min. The gas stream was sterilized by filtration. The sterile medium was fed at the bottom of the reactor by means of a peristaltic pump.

Tygon rings were selected as carriers. No chemical was used to assist cell immobilization on the selected carrier (Raganati et al., 2013).

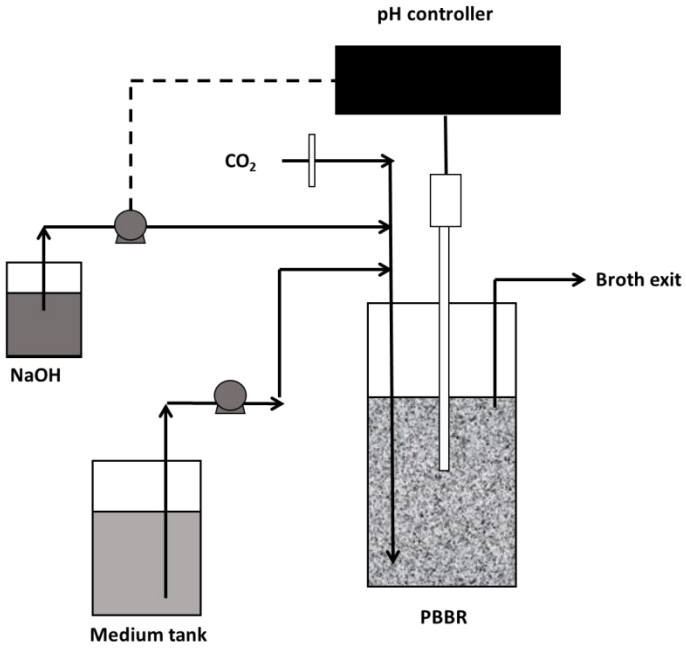

Figure 4.2.1: *Outline of the apparatus or continuous tests.*

Analytical methods

Cell density was measured as optical absorbance at 660 nm (OD_{660}) by using a spectrophotometer (Cary- 50 Varian).

The concentration of soluble species was measured in the liquid phase after spinning down the cells by centrifugation (13000g, 10min). Sugar and organic acid concentrations were measured by means of a high-performance liquid chromatography (HPLC) (HP1250 working station system - Agilent Technologies, USA) equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm×7.8 mm, 9 µm; Bio-Rad Chemical Division, Richmond, CA). Analytes were detected by UV absorbance (Agilent Technologies, G1315D) and refractive index (Agilent Technologies, G1362A). H_2SO_4 5mM was used as mobile phase at 0.6 mL/min flow rate at room temperature. The injection volume was 20 µL.

Experimental procedures and data analyses

300 µL of stock culture was transferred in 10 mL Hungate tubes containing the culture media (37 g/L of BHI broth). The cultures were incubated for 24 hours under anaerobic batch conditions, then 30 mL of active culture were inoculated in the reactor.

Tests aimed at succinic production were carried out with the packed bed biofilm reactor (PBBR) operated at pre-set conditions. 34.4 g Tygon rings used to prepare a 4.5 cm high packed bed. The volume of the reactor was set at 40 mL by means of the overflow duct.

The start-up of the biofilm in the PBBR was carried out according to the procedure reported by Napoli et al., (2010)

The dilution rate (D) – the ratio between the feeding volumetric flow rate and the volume of the fixed bed – ranged between 0.64 and 2.4 h^{-1}. The biofilm reactor performances were assessed by measuring the concentration of sugar(s) and metabolites provided that the steady-state had stabilized – concentration of all metabolites and sugars constant - for at least 10 times the reactors mean residence time (1/D). Reactor

performances were reported in terms of sugar conversion degree (ξ_S), sugar-to-‘‘i-species’’ fractional yield coefficient ($Y_{i/S}$), succinic acid productivity (W_{SA}) and succinic acid selectivity with respect to the other acids (χ_{SA}). ξ_S, $Y_{i/S}$, W_{SA} and χ_{SA} were assessed assuming that: (i) the feeding was aseptic and free of metabolites, and (ii) the gas stripping of metabolites was negligible. According to these assumptions be means of Eq.s (4.2.1) trough (4.2.4):

$$\xi_S = \frac{S_{IN} - S_{OUT}}{S_{OUT}} \quad (4.2.1)$$

$$Y_{i/S} = \frac{i_{OUT}}{S_{IN} - S_{OUT}} \quad (4.2.2)$$

$$W_{SA} = D \cdot SA_{OUT} \quad (4.2.3)$$

$$\chi_{SA} = \frac{D \cdot SA_{OUT}}{D \cdot (SA_{OUT} + AA_{OUT} + FA_{OUT})} \quad (4.2.4)$$

where S, SA, AA, FA and “i" are the concentration of sugar, succinic acid, acetic acid, formic acid and generic metabolites, respectively, measured in the feeding (suffix IN) and in the effluent (suffix OUT).

The mass of biofilm in the reactor was assessed at the end of the run in agreement with the following procedure: (i) the dry carrier was weighted before filling the reactor; (ii) at the end of the test the reactor was rinsed with sterile water to remove sugars and metabolites; (iii) the carriers with the biofilm were harvested and dried at 40 °C for 24h, and (iv) the dried mass of the biomass and carriers were weighted. The dried mass of the biofilm in the reactor was assessed as the difference between the weight of the carrier-biofilm and the carriers.

Design of experiments

The tests were aimed to assess the performance of the PBBR under a wide range of dilution rate and by feeding the reactor with stream bearing a spectrum of substrate

The first set of experiments was carried out by feeding the PBBR, with a glucose-based medium (glucose concentration set at 50 g/L). and the dilution rate was set between 0.5 and 2.4 h^{-1}.

The second set was of experiments was aimed to adapt the cells to a xylose-based medium, the main pentose sugar present in a lignocellulosic hydrolysate. The feeding was a solution of glucose and xylose at percentage of xylose progressively increased from 0 up to 100%. The total sugar concentration (glucose+xylose) in the feeding was set at 50 g/L. The dilution rate was set at 1.24 h^{-1}.

After evaluating how the PBR performances changed increasing the xylose concentration in the media, the effect of the dilution rate on the succinic acid production by using a xylose based medium was investigated. For this set of experiments the xylose concentration was set to 40 g/L and the dilution rate was set between 0.5 h^{-1} to 1.44 h^{-1}.

The fourth set of experiments was carried out by feeding the PBBR with a synthetic medium that mime the composition of a lignocellulosic hydrolysate (inhibitor free). The feeding was a solution of glucose, arabinose and xylose (GAX): the total sugar concentration was set at 80 g/L and the mass ratio between the sugars was set to at 55:15:30 (Li et al., 2011). The dilution rate was set between 0.7 h^{-1} and 1.44 h^{-1}.

The last set of experiments was carried out to investigate the effects of the two principal inhibitors, furfural and HMF (Palmqvist and Hahn-Hagerdal, 2000), found in

lignocellulosic hydrolysate on the fermentation process. The concentration of the inhibitors in the feeding was set at 1g/L and 0.28 g/L (Martinez et al., 2001), for furfural and HMF respectively. The dilution rate was set at 0.75 and 1.00 h^{-1}.

Results

Biofilm start-up

The PBBR was inoculated with actively growing cells at t=0 and operated in batch mode with respect to the liquid phase for 24 h after (data not shown). After 24 h, the PBBR was switched to continuous mode feeding 40 g/L glucose: medium (synthetic medium), setting the dilution rate was set at 0.20 h^{-1}. A visible biofilm layer formed on the carriers in about 3 days and at t=7 day the dilution rate was increased up to 0.84 h^{-1} to promote the biofilm production over the suspended cell growth. Figure 4.2.2 reports the time series of the concentration of acids and glucose during the start-up of the PBBR.

At t=16 days the carriers were covered with abundant biofilm and steady state conditions had established in the reactor. Altogether, the biofilm reactor start-up took about 17 days and a remarkable amount of active biofilm was formed. The suspended biomass detected under steady-state conditions was very low and a clear effluent was observed for D larger than 0.84 h^{-1}.

Provided the stable formation of the biofilm, the pH in the reactor was controlled. The pH was set at a value slightly higher than the optimal value reported in literature (Van der Werf et al., 1997) because pH and metabolites gradient was expected across the biofilm (Qureshi et al., 2005). As a consequence the pH within the biofilm was expected to be lower than that measured in the broth.

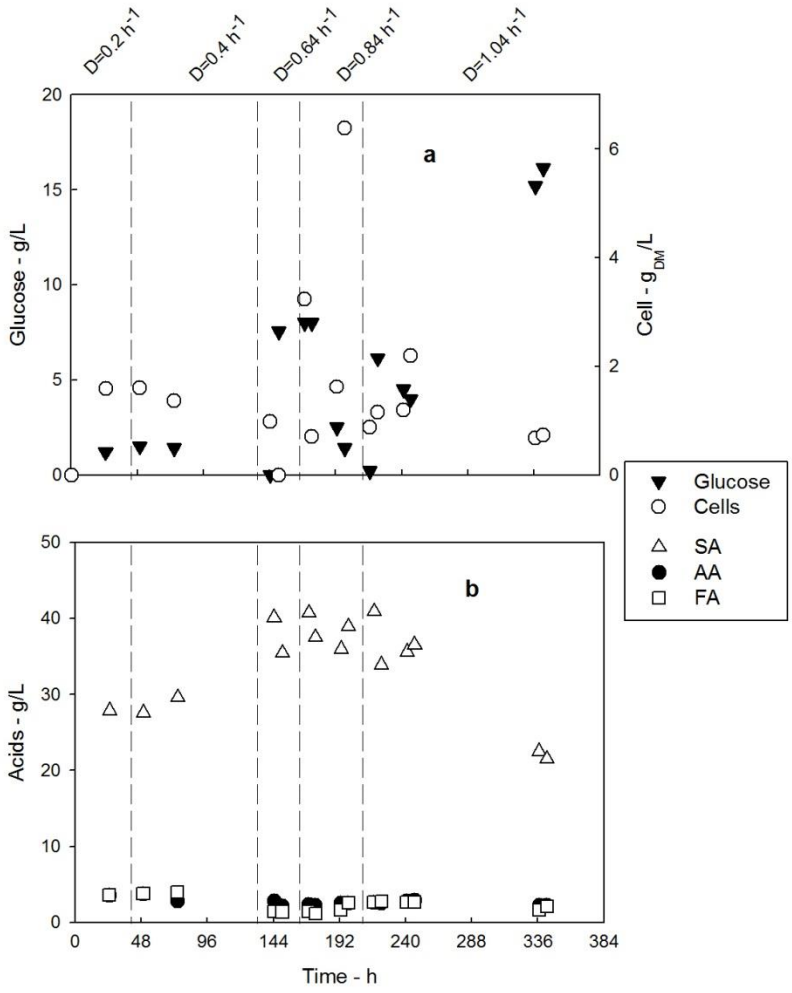

***Figure 4.2.2**. Main data measured during PBBR start-up. The vertical dotted line marks the changes of the dilution rate. a) cells and glucose concentration b) acids concentration.*

Continuous SA production

Glucose as carbon source

At t= 17 days the succinic acid production started. The dilution rate was set at 0.5 h^{-1} and the steady state was characterized. The D was increased of 0.2 h^{-1} every time to establish a new steady - state.

The system approached a steady-state regime within 2 to 4 days, depending on the dilution rate set. PBBR performance was characterized in terms of metabolite concentration, glucose conversion degree, succinic acid yield, productivity and selectivity. Data were assessed by processing the dilution rate and the concentration of glucose and metabolites as reported in the Section 2.5. Data reported in Table 4.2.1 were assessed under steady - state conditions: the concentration of the sugar and of the metabolites were constant for at least 10 times the reactor mean residence time ($\tau = 1/D$). The effect of the dilution rate (D) on the performance of the PBBR was investigated.

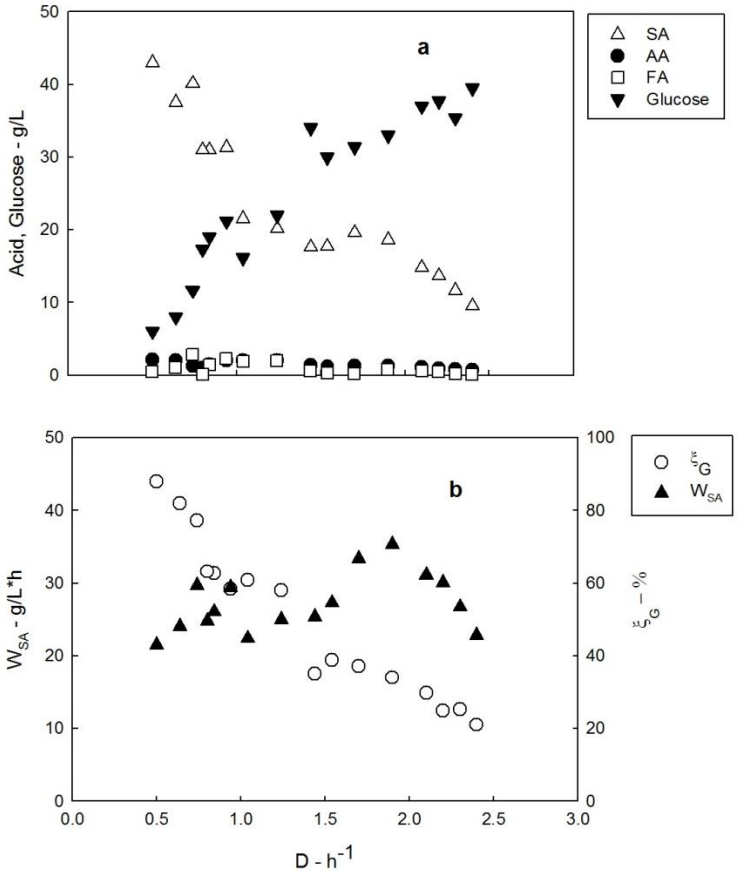

Figure 4.2.3. *Data measured during the PBBR operation as a function of the dilution rate using a glucose-based medium (50 g/L). a) concentration of glucose and acids b) succinic acid productivity and glucose conversion.*

Figure 4.2.3 and Table 4.2.1 report the main data measured during the continuous fermentation as a function of the dilution rate. The dilution rate was steadily increased from 0.84 to 2.4 h^{-1}. The analysis of the results reported in Figure 4.2.3 and Table 4.2.1 highlighted the issues reported hereinafter:

- the glucose conversion degree (ξ_G) and produced succinic acid concentration significantly decreased with D. They were characterized by a maximum at the lower investigated D;
- succinic acid productivity was characterized by a maximum (35 g/L*h) at D= 1.9 h^{-1}. It is worthy to note that the SA productivity was the largest values reported in the literature.
- The concentration of acetic and formic acid was always below 5 g/L. Succinic acid selectivity was quite high and about constant with D (ranging between 0.84 and 0.96 g/g).

Xylose as carbon source

PBBR performance feeding a synthetic medium bearing xylose was investigated. as xylose represents the main pentose sugar found in lignocellulosic biomass hydrolysate (Schell et al., 2003; Weiss et al., 2010). A test campaign was addressed to adapt the cells to the new sugar: tests with medium containing both glucose and xylose (GX medium) were carried out. The fraction of xylose was progressively increased from 0 to 100% and the dilution rate was set at 1.24 h^{-1}.

In Figure 4.2.4a, the concentration of the acids and the succinic acid productivity are reported as a function of the sugar composition of the medium (percentage of xylose). Succinic acid concentration and productivity decreased with xylose fraction in the feed. The observed behaviour of SA production is in agreements with the results of tests carried out under batch conditions: *A. succinogenes* metabolizes glucose better than xylose (M. Ferone et al., 2017b). It interesting to note that increasing the percentage of xylose in the media acetic and formic acid production also increase and the SA selectivity decreased.

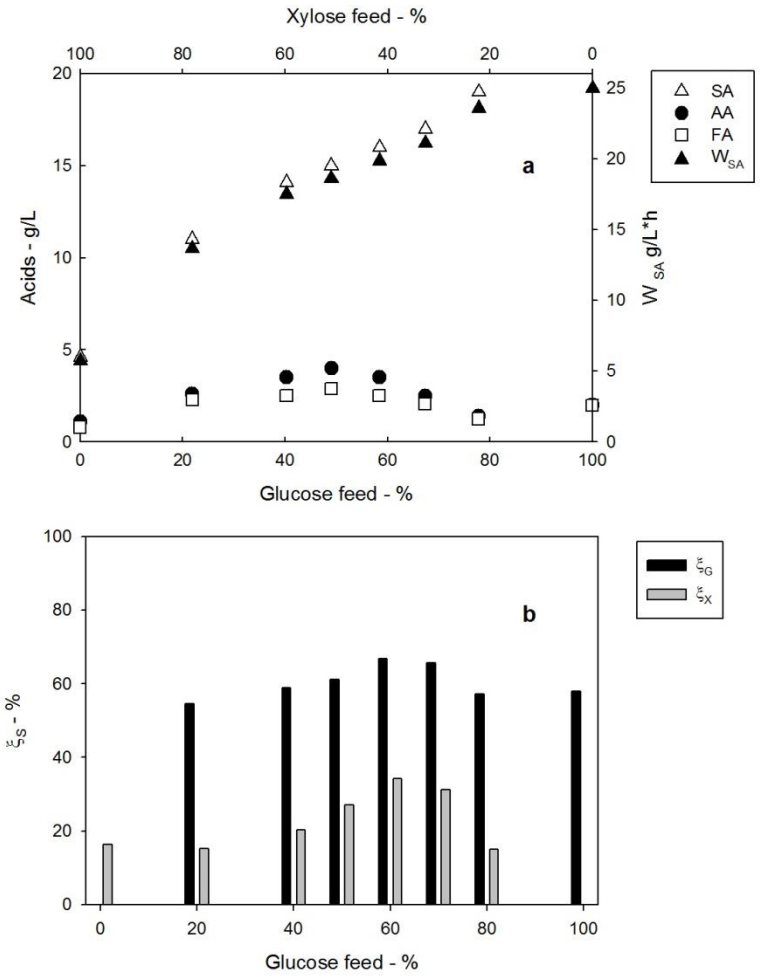

Figure 4.2.4. *Data measured during the PBBR operation as a function of the dilution rate using a glucose-xylose based medium (total sugar concentration 50 g/L). a) concentration of acids and succinic acid productivity b) sugars conversion degree.*

Figure 4.2.4b reports sugar conversion as function of percentage of xylose in the feeding. Glucose conversion is constant (around 60%) whereas xylose conversion was characterized by a maximum (35%) when the xylose concentration in the feeding is about 40%.

As the biofilm was adapted to the xylose, the third set of experiments started, feeding the PBBR with a xylose-based medium (xylose concentration set at 40 g/L); the dilution rate was set between 0.5 h^{-1} to 1.44 h^{-1}. The steady - states were characterized in terms of acids concentration and xylose conversion degree.

As reported in Figure 4.2.5, succinic acid concentration and xylose conversion decreased with the dilution rate . These results were expected as an effect of the reduced residence time in the bioreactor.

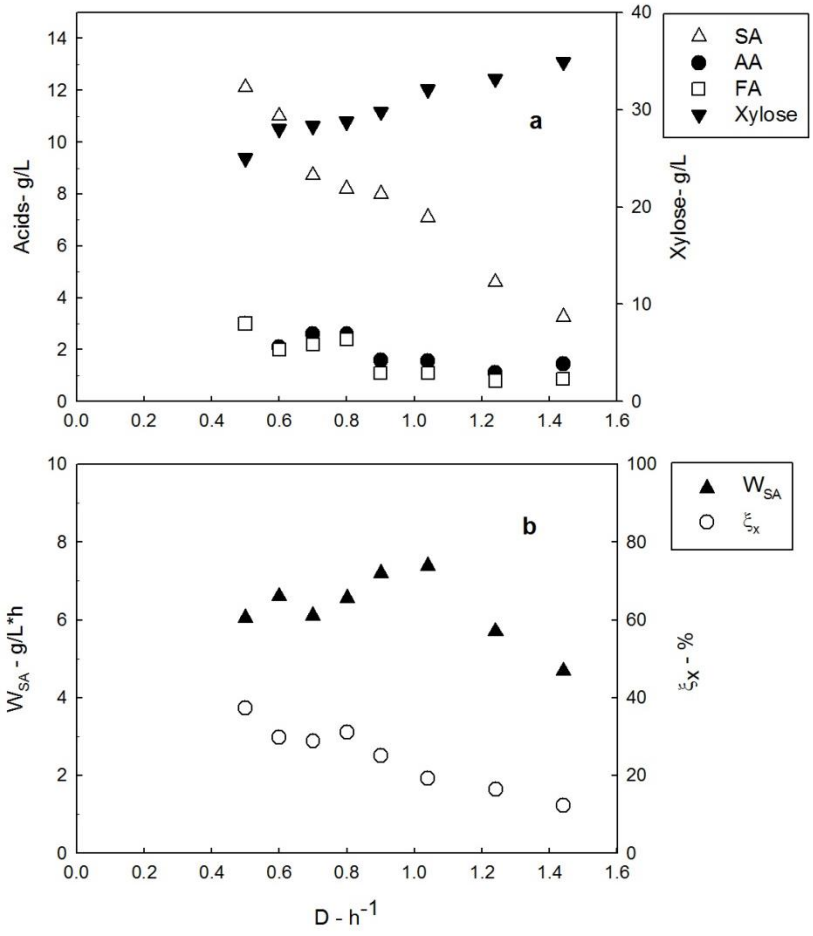

Figure 4.2.5*: Data measured during the PBBR operation as a function of the dilution rate using a xylose-based medium (40 g/L). a) concentration of xylose and acids b) succinic acid productivity and xylose conversion.*

Succinic acid productivity was quite low when using xylose as the sole carbon source: it reached the maximum value 7.38 g/L*h at D = 1.04 h^{-1}. However, the productivity is still interesting when compared with results available in the literature. Bradfield and Nicol (2015) reported succinic acid production from pure xylose by *A. succinogenes* biofilm and they found that the production was lower than 4 g/L*h at the three investigated dilution rate (D= 0.05, 0.10 and 0.30 h^{-1}).

Glucose- Arabinose-Xylose (GAX) as carbon source

The PBBR performances were evaluated for test carried out feeding the bioreactor with a synthetic lignocellulosic hydrolysate (inhibitor free) containing glucose, arabinose and xylose. The total sugars concentration in the synthetic medium was set at 80 g/L and the mass ratio between the sugars was 55:15:30 for the GAX mixture (Li et al., 2011). The dilution rate ranged between 0.7 h^{-1} and 1.44 h^{-1} and each steady-state condition was characterized in terms of acid concentration and sugar conversion degrees. Figure 4.2.6a reports that the succinic acid concentration decreased with the dilution rate: the maximum SA concentration was 20.5 g/L at D=0.7 h^{-1}. As regards the sugar conversion degrees (Figure 4.2.6b), glucose and xylose conversion degrees decreased with the D, and the

arabinose conversion degree varied between 3% to 20%. The total sugar conversion degree also decreased with D.

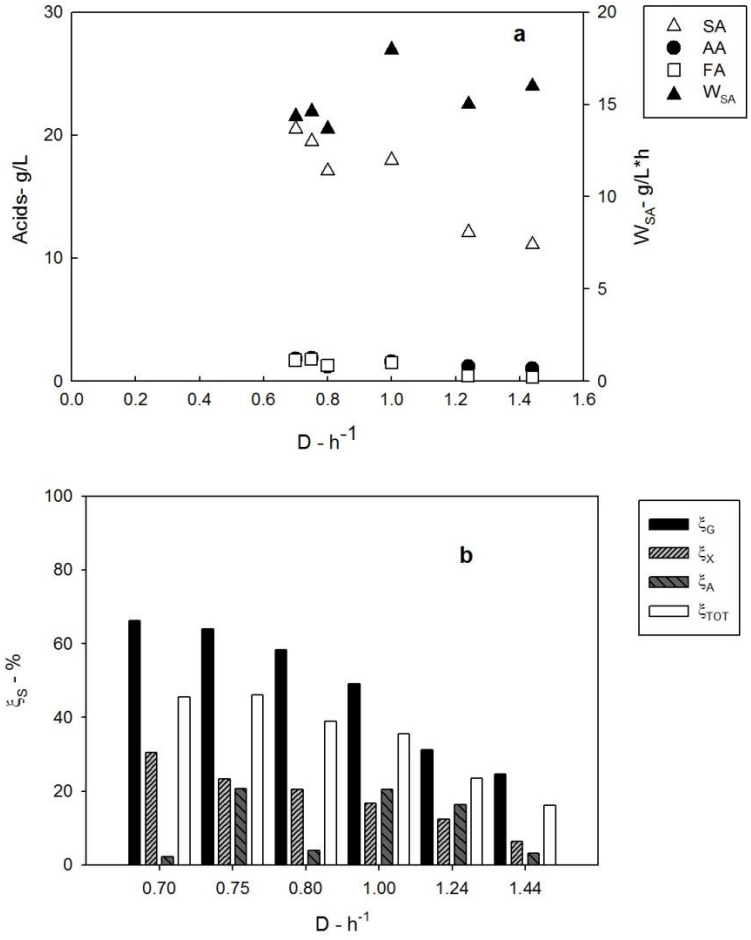

Figure 4.2.6*:Data measured during the PBBR operation as a function of the dilution rate: glucose-arabinose-xylose based medium. Total sugar concentration 80 g/L; GAX mass ratio 55:15:30. a) concentration of acids and succinic acid productivity b) sugars conversion.*

The succinic acid productivity was fairly constant with D and it was about 15 g/ L*h. The PBBR performances expressed in terms of SA concentration and productivity were slightly lower in the tests carried out with GAX solution (Figure 4.2.6) than that measured in tests with GX solution (Figure 4.2.4): at the same dilution rate (D= 1.24 h^{-1}) and similar glucose and xylose fraction (about 60% glucose 30% xylose) in the feeding: the SA concentration and productivity decreased when arabinose was present.

Effect of inhibitors on succinic acid production

The effects of the two potential byproducts of the lignocellulosic hydrolysate - furfural and HMF – typically acting as fermentation inhibitors (Palmqvist and Hahn-Hagerdal, 2000) were investigated. The succinic acid production by *Actinobacillus* biofilm was characterized during the feeding of a GAX solution supplemented with 1g/L furfural and 0.28 g/L (Martinez et al., 2001), HMF. They were added separately into the GAX medium and their effect was evaluated at two different dilution rates (0.75 and 1.00 h^{-1}).

Table 4.2.1 reports the main data measured/calculated for the fermentation tests carried out by supplementing furfural and HMF to the GAX medium. The concentration of produced succinic acid decreased with respect to the inhibitor free medium as inhibitors were supplemented. The SA concentration decreased of about 5.6% and 16% (at D= 0.75

and 1.0 h^{-1}, respectively) in presence of furfural, while the concentration of SA produced was reduced by 10.8% and 22.6 % when HMF was supplemented ,compared to the inhibitor-free GAX medium at same operating conditions.

The SA selectivity was not affected by the presence of the inhibitors. This result would suggest that the flux distribution between the C4 and the C3 pathway (Mckinlay et al., 2005) does not change as inhibitors are present in the medium.

The biofilm PBBR was stopped at the end of the run with the inhibitors, after 5 months of continuous operation. and the overall biomass concentration was 107 g_{DM}/L.

***Table 4.2.2**: Main results of fermentation test adding furfural or HMF to the GAX medium, compared to the inhibitor-free GAX tests.*

D – h^{-1}	**0.75**			**1.00**		
	GAX	*GAX + Furfural*	*GAX + HMF*	*GAX*	*GAX + Furfural*	*GAX + HMF*
SA - g/L	19.50	18.40	17.40	17.96	15.10	13.90
ξ_{TOT} - %	46.00	50.97	43.40	35.45	35.83	35.21
W_{SA} – g/L*h	14.60	13.80	13.05	17.96	15.10	13.90
Y_{SA} – g_{SA}/g_S	0.55	0.50	0.56	0.65	0.58	0.55
χ_{SA} g_{SA}/g_{TotAc}	0.84	0.75	0.84	0.85	0.86	0.87

Discussion

Succinic acid is well established as bio-based platform chemical with production quantities expecting to increase exponentially within the next decade. *Actinobacillus succinogenes* is by far the most studied wild type succinic acid producing microorganism and the most interesting for industrial applications. Despite the requirement for high productivities to reduce the production costs, the majority of the literature publications are focused on batch fermenters typically characterized by low productivity and long dead-time. From a processing perspective, high cell density fermentation could enhance volumetric productivity and reduce capital costs. Given the economic requirement for high cell density fermentation, more insight is required on the rate and yield characteristics of *A. succinogenes* biofilms.

To this respect, the results reported in the present study are very promising when compared to previous investigation regarding SA production by *A. succinogenes*. In particular, in the present investigation was obtained the highest productivity among that reported in the literature: 35.0 g/L*h for glucose fermentation. The optimal results were obtained at the dilution rate 0.5 h^{-1}: 43.0 g/L of succinic acid were produced, glucose conversion was 88%; and the volumetric productivity was 22 g/L*h, still higher than that reported in literature.

The possibility to produce succinic acid from different sugars by *A. succinogenes* is in agreement with previous investigations (M. Ferone et al., 2017b): the bacterium could simultaneously uptake glucose, mannose, arabinose and xylose to produce succinic acid. Therefore it was expected to have the co-fermentation of the sugar present in the synthetic lignocellulosic hydrolysate medium (GAX medium) by *A. succinogenes* biofilm.

It is worth to note to compare the results reported in the present investigation with those reported by Salvachúa et al., (2016). They used a custom continuous fermentation setup to produce SA from corn stover hydrolysate stream, containing xylose, glucose, arabinose, and galactose, produced from deacetylation and dilute acid pretreatment. The maximum SA concentration, yield and productivity were 39.6 g/L, 0.78 g/g and 1.77 g/L*h, respectively, at a dilution rate of 0.05 h^{-1}. Despite the low SA concentration measured

during the tests carried out with the GAX solution (Figure 4.2.6), the productivity was almost one order of magnitude larger than that reported by Salvachúa et al. (2016). To the author knowledge, this is the only study available in the scientific literature regarding the continuous SA production from lignocellulosic hydrolysate by *Actinobacillus* biofilm. Further comparison with SA production studies by using *A. succinogenes* and biomass feedstockes may be proposed only with reference to batch fermentation mode.

The effect of the primary suspected fermentation inhibitors, furfural and HMF, was also investigated in this study. It was found that both the inhibitors reduced succinic acid production when compared with results of the tests with an inhibitor-free GAX medium. The HMF had a stronger inhibiting effect compared to furfural. However, it should be pointed out that the concentration of the inhibitor species was not monitored during the tests; therefore, it is not known if there was any conversion of the inhibitors. As suggested in a previous study (Ran et al., 2014), furfural can be converted to furfuryl alcohol by means of an aldehyde reductase because the aldehyde may be reduced to its alcohol form. Moreover, the genome of *A. succinogenes* encodes an aldo/keto reductase (McKinlay et al., 2010) that may be responsible for the reduction of furfural.

Conclusions

Succinic acid production by *A. succinogenes* fermentation in a packed bed reactor (PBBR) was successfully carried out for more than 5 months. The effects of the dilution rate (D) and of medium composition (glucose, GX, xylose, GAX media) on the PBBR performances were investigated. Succinic acid concentration, productivity and sugar(s) conversion generally decreased with D. A maximum succinic acid productivity of 35.0 g/L*h was achieved at D= 1.9 h^{-1}. The effect of two inhibitors was also investigated. HMF remarkably reduced succinic acid production when compared to furfural.

Acknowledgements

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***Table 4.2.1**: Biofilm steady-state results obtained during fermentation tests carried out with glucose, xylose and GAX media*

Medium	D h^{-1}	SA g/L	ξ_S %	$Y_{SA/S}$ g_{SA}/g_S	χ_{SA} g/g
Glucose	0.64	37.5	81.9	1.03	0.92
	0.84	31.0	62.7	0.97	0.91
	1.04	21.5	60.8	0.86	0.85
	1.24	20.1	57.9	0.66	0.83
	1.44	17.6	35.0	0.96	0.90
	0.94	31.3	58.4	1.05	0.88
	1.54	17.7	38.8	0.93	0.92
	1.70	19.6	37.2	1.05	0.93
	1.90	18.6	34.0	1.09	0.90
	0.50	43.0	88.0	0.98	0.94
	0.74	40.1	77.1	1.02	0.91
	0.80	31.0	63.2	1.04	0.97
	2.10	14.8	29.8	0.94	0.90
	2.20	13.7	24.9	1.09	0.91
	2.30	11.6	25.3	0.97	0.92
	2.40	9.5	21.0	0.90	0.92
Xylose	0.80	8.2	31.1	0.63	0.62
	0.90	8.0	25.0	0.80	0.75
	1.24	4.6	16.5	0.70	0.71
	1.44	3.3	12.2	0.67	0.58
	1.04	7.1	19.2	0.93	0.73
	0.70	8.7	28.8	0.76	0.64
	0.60	11.0	29.8	0.92	0.73
	0.50	12.1	37.3	0.81	0.67
GAX	0.80	17.1	39.0	0.57	0.87
	1.00	18.0	35.5	0.65	0.85
	0.70	20.5	45.6	0.56	0.85
	1.24	12.1	23.5	0.64	0.88
	1.44	11.1	16.2	0.88	0.89
	0.75	19.5	46.0	0.55	0.84

5 DISCUSSION

Since many years petroleum oil has been used both as a source of fuels and to produce chemicals and materials. Nowadays, world average annual demand of the petroleum for chemical purposes is over one billion barrels and overall consumption of liquid fuels such as crude oil and natural gas is over 30 billion barrels per year. Petrochemical route is very competitive due to the continuous optimization and researches on the process for more than half of a century as compared to biological approach and the (low) cost of the feedstock. On the contrary, biobased chemical production is challenging due to the fact that it is in the developing phase and it requires innovative operations to support the success of the biorefinery route. However, the biorefinery approach to produce biobased products may become competitive with respect to the petroleum route because the depletion of fossil resources is in progress (years or centuries, it happens!) and the optimisation of biotechnological processes is in progress too. Various types of chemicals that are conventionally produced by chemical processes could potentially be generated via biotechnological processes using biological materials as feedstocks and micro-organisms as biocatalysts (Ragauskas et al., 2006).

Succinic acid has been recognized by the US Department of Energy as one of the 12 top value-added chemicals that can be produced from biomass in 2004 (Werpy and Petersen, 2004). Bio-based succinate production may replace the petrochemical-based route with marked environmental benefits: 1) the use of renewable feedstock as substrate for the fermentation (Salvachúa et al., 2016b) and 2) CO_2 is fixed into succinic acid during the fermentation (Van der Werf et al., 1997). Recognizing its potential importance, extensive research has been carried out in the world.

Bioprocessing costs and environmental impact are highly dependent on three main issues: i) the selection of the raw material and the upstream pre-treatment stages required to produce a nutrient-complete fermentation medium; ii) the fermentation stage; iii) the downstream separation and purification of succinic acid. This study addressed the first and the second mentioned issues.

5.1 Renewable feedstocks for SA production

Feedstocks typically utilized for large scale fermentative production of organic acids are refined sugars (sucrose, glucose and fructose), starch and beet or cane molasses**.** The use of agriculture and forest related residues, industrial waste and by-product streams have a high potential as an alternative and sustainable source of raw material for chemical industries.

Lignocellulosic biomass have been extensively investigated for succinic acid production (Akhtar et al., 2014). Pretreatment of lignocellulosic biomass should be carried out via combined thermo-chemical and enzymatic treatment in order to produce C5 and C6 sugars.

Chen et al. (2011) reported the production of 35.4 g/L succinic acid at yield of 0.72 g/g and productivity of 0.98 g/L*h by the fermentation of corn hydrolysates, produced via sulfuric acid pretreatment, followed by $CaCO_3$ neutralization and activated carbon absorption (targeting the removal of furfural). Liu et al., (2008) utilized cane molasses as a low-cost carbon source to produce succinic acid by *A. succinogenes* CGMCC1593. In batch fermentation, 46.4 g/L of succinic acid with the yield of 79.5% was attained in 48 hours.

Jiang et al., (2013) reported succinic acid production from a sugar mixture - composed of cellobiose, glucose, arabinose, and xylose, obtaining 21.7 g/L of

succinic acid and a yield of 0.68 g_{SA}/g_S. However, they also measured a concentration of acetic acid of about 15 g/L, higher than that measured during the present investigation (about 6 g/L). According to the reported results the SA specificity was lower than that reported in the present investigation. Succinic acid fermentation from sugar mixtures was also reported by Li et al., (2010). The sugar mixtures, derived from the hydrolysis of corn stalks and it contained mainly glucose, xylose, and cellobiose. They produced 17.8 g/L of succinic acid at yield of 0.66 g_{SA}/g_S. In both these studies, higher productivities were achieved (0.66 g/L h), since the fermentation times was quite short, 35 and 40 h, respectively.

The yield obtained in the present study (Ferone et al., 2016, Ferone et al., 2017a) is generally higher than that reported in the literature when the comparison is with data from fermentation carried out under close operating conditions: batch fermentation and same sugar.

Results of investigations carried out using lignocellulosic hydrolysate as feedstock of continuous fermentaters to produce succinic acid are available in the literature. Bradfield et al., (2015) used a custom continuous fermentation setup to produce succinic acid from corn stover hydrolysate stream, containing xylose, glucose, arabinose, galactose, produced from deacetylation and dilute acid pretreatment. The maximum succinic acid concentration, yield, and productivity were 40 g/L, 0.78 g/g and 1.77 g/L*h, respectively, were achieved, at a dilution rate of 0.05 h^{-1}.

In Ferone et al. (2017d) was reported the continuous succinic acid production in a biofilm packed bed reactor. The maximum SA concentration obtained when the reactor was feed with a medium that mime the composition of a lignocellulosic hydrolizate, was 20.5 g/L at D=0.7 h^{-1}, corresponding to a succinic acid productivity of 14.4 g/ L*h.

Waste and by-product streams from the food and beverages industries could also be used for the production of succinic acid. Ferone et al., (2017b) (section 3.3) reported about 40 g/L of succinic acid produced using high content sugar beverages as carbon source, without the need for additional nutrient supplement in the media when using fruit juices.

5.1.1 Detoxification of lignocellulosic hydrolisates

Although lignocellulosic biomass are environmental-friendly and cost advantage feedstocks compared with pure sugars several inhibitors are formed during pretreatment. The main inhibitors compounds formed during the pretreatment of lignocellulosic biomass are: weak acids (acetic, formic and levulinic acid), HMF (hydrossimethylfurfural) and furfural, phenolic compounds (4-hydroxybenzoic acid, vanillin, syringaldehyde) (Palmqvist and Hahn-Hagerdal, 2000). These inhibitors cause the decrease of cell growth rate and productivity, which requires additional costs in the purification process. The severity of different pretreatment conditions (temperature, time and pH) influence the generation of fermentation inhibitors (Pienkos and Zhang, 2009).

Several different chemical, biological and physical methods to detoxify slurries and hydrolysates have been investigated (Cantarella et al., 2004; Larsson et al., 1999).

One of the possibility is to use less recalcitrant feedstocks and to utilize mild pretreatment conditions, but this strategy would lead to a poor sugar yield, which is

not desirable. Other strategies are based on the fermenting microorganism. For example, the use of a larger inoculum (Parawira and Tekere, 2011) might help decreasing inhibitions problems, but is not convenient form the industrial point of view. Another possibility is the selection of microbial species and strains that exhibit resistance to inhibitors, through adaptation or genetic engineering (Adeboye et al., 2016; Narayanan et al., 2016).The different procedures that have been studied to detoxifying lignocellulosic hydrolysates include treatments with chemicals, liquid-liquid extraction, liquid–solid extraction, heating and evaporation, and treatments with microbial and enzymatic biocatalysts. In particular, detoxification by addition of alkali (such as calcium or sodium hydroxide), has emerged as one of the most efficient and economic methods. However, a drawback of this detoxification strategy is that also the sugars are affected by the treatment.

An emerging technology is the possibility to perform the detoxification *in situ* in the bioreactor by using reducing agents, such as sulfur oxyanions or sulfhydryl reagents (Alriksson et al., 2011), eliminating the need for an extra process step. Furthermore, treatment with reducing agents also reduce the enzymes inhibition during the hydrolysis.

5.2 Fermentation stage optimization

Kinetic models are key tools in developing a fermentation process to support the optimization of the operating conditions to maximize the production of target metabolites. Both structured and unstructured models have been used for kinetic modelling. Although the former class of models can describe complex microbial systems at the molecular levels, the latter class of models - unstructured kinetic models - have frequently been used for practical applications (Moran et al., 2007; Russo et al., 2008). Some studies are present in literature on the kinetic characterization of *A. succinogenes* growth and succinic acid production on various substrate, but they are typically based on experimental data measured during batch fermentations.

Lin et al. (2008) reported an unstructured model to describe succinic acid production by *A. succinogenes* cultivated on glucose and wheat hydrolysates. Corona-González et al., (2008) also reported succinic acid production from glucose with *A. succinogenes ZT-130* and estimated kinetic parameters with glucose as carbon source. The succinic acid production from glycerol was simulated by Vlysidis et al. (2008) using a modified Monod equation and considered both substrate and product inhibition to predict the behaviour of the main variables at various initial glycerol concentrations. Pateraki et al. (2016) proposed an unstructured model to predict the cultivation of *A. succinogenes* and *B. succiniciproducens* on a mixture of C5 and C6 sugars: , similar to the sugar composition of spent sulphite liquor, the liquid waste stream from the sulphite pulping process.

The present study (Ferone et al., 2017c) – section 4.1 - proposes a kinetic model and the assessment of the kinetic parameters for succinic acid production under continuous operating conditions. The results obtained suggest that, under the operating conditions tested, no glucose inhibition occurred and that acetic acid just barely inhibited the cell growth.

Continuous bioconversion in immobilized cell reactors has several advantages with respect batch cultures (Qureshi et al., 2005), the fermentation mode typically used for succinic acid production. The main advantages are: i) high cell concentration; ii) ease separation of cells from products; iii) high productivity.

A. succinogenes is well known to self-adhere to support surfaces and form biofilms under prolonged operation. Indeed, continuous studies on *A. succinogenes* resulted in unavoidable biofilm formation (Bradfield and Nicol, 2014; Urbance et al., 2004; van Heerden and Nicol, 2013).

In Corona-González et al. (2014), the production of succinic acid with *A. succinogenes* entrapped in agar beads was studied. The succinic acid concentration of 43.4 g/L was obtained from 78 g/L glucose, corresponding to a volumetric productivity of 0.68 g/ L*h.

Continuous anaerobic fermentations in a biofilm reactor packed with Poraver® beads were carried out by Maharaj et al. (2014). They reported a volumetric productivity of 10.8 g/L*h at D=0.7 h^{-1} using a glucose-based medium.

The highest productivity reported in literature was obtained by Brink and Nicol (2014). They reported a productivity of 17.1 g/L*h at D=2.2 h^{-1} using a novel shear controlled fermenter, that enabled both chemostat and biofilm operation.

Bradfield et al. (2015) used a custom biofilm reactor to investigate the succinic acid production from pure xylose by *A. succinogenes.* The yields (0.55–0.68 g/g), titres (10.9–29.4 g/L) and productivities (1.5–3.4 g/L*h) were lower than those obtained in previous studies on glucose, but product ratios (succinic acid/acetic acid) and carbohydrate conversion rates were similar.

During the tests carried out in the present study (Ferone et al., 2017d) – section 4.2 – the measured succing acid production was the highest reported in the literature for *A. succinogenes* fermentations. The succinic acid production by *A. succinogenes* fermentation in a packed bed reactor (PBBR) was characterized by productivity of 35 g/L*h at D = 1.9 h^{-1} by feeding the reactor with a glucose bearing stream. Succinic acid productivity was much lower when feeding the PBBR with xylose as the sole carbon source: the maximum productivity was 7.38 g/L*h at D=1.04 h^{-1}.

5.3 Future perspectives for bio-based SA production

Considering the extremely low prices required for bulk chemicals to be successful on the market, investments in equipments as well as the operating costs of the industrial production process need to be extremely low. The cost of substrate is a crucial features for a process to become economically viable. This is particularly true for commodity chemicals for which the price might be only slightly higher than the price for refined sugar. Lignocellulosic biomass and industrial waste streams have been shown to be very promising feedstocks for succinic acid production on industrial scale.

The producion of succinic acid as a bulk bio-based platform chemicals requires the construction of industrial plants including bioreactors characterized by high throughput. Therefore, the use of facultative anaerobic microorganisms – e.g. *Actinobacillus succinogenes* - reduces bioreactor costs due to the absence of aeration that increases significantly capital and operating costs.

To keep the productivity at reasonable values a potential solution is confinement of cells in the reactor to increase cell concentration. The immobilization of the microbial cells is a solution. Moreover, it has the additional advantage of facilitating their reuse.

In the present study, a packed bed biofilm reactor was developed for succinic acid production by *A. succinogenes* from a synthetic lignocellulosic biomass hydrolizate. It allowed to obtain much higher productivity compared to the suspended cells systems typically employed.

Three-phase fluidized bed bioreactors are excellent candidates for bioconversion processes (Schugerl, 1997) with a wide variety of reactor typologies available. Airlift bioreactors are a low price alternative to stirred tank reactors and could potentially have an important role in microbial bulk production processes in the future. For instance, Liu et al., (2006) successfully used airlift bioreactors for a scale-up of lactic acid production with *R. oryzae*.

Although several downstream separation methods have been suggested so far, the efficient purification of succinic acid is still considered as a critical parameter for the application of the process at large scale. Lower pH values of the fermentation broth reduce the downstream separation cost as the pH value affects the dissociation level of succinic acid ($pK_a1 = 4.16$ and $pK_a2 = 5.6$).

Finally, the integration of bio-based succinic acid production into existing industrial facilities and the development of integrated biorefineries will lead to significant economic and environmental benefits.

In the following sections, downstream process for succinic acid recovery and genetic engineering approaches as a strategy to improve the fermentation performances are discussed. Finally, a biorefinery approach for succinic acid production is proposed.

5.3.1 Downstream process for SA

In the biotechnological process to produce succinic acid, about 60% of the total production costs are generated by downstream processing, e.g. the isolation and the purification of the product in the fermentation broth (Bechthold et al., 2008). Several methods have been proposed to purify succinic acid from the fermentation broth. The first downstream processing step is cell separation by centrifugal separation or microfiltration, usually followed by ultrafiltration to separate cell debris, proteins and other polymers from the fermentation supernatant.

The conventional separation process of organic acids employed by the traditional fermentation industry is the precipitation with calcium hydroxide (Berglund et al., 1991; Datta et al., 1992). After addition of calcium hydroxide or calcium oxide, calcium succinate is recovered by filtration and converted into succinate with the addition of concentrated sulfuric acid. The free succinic acid is purified by some commonly applied methods, such as active carbon or ion exchange and then the product is further concentrated and crystallized by evaporation. However, the purification of succinic acid by precipitation with calcium hydroxide presents significant disadvantages, such as the equimolar production of calcium sulfate, high demand for calcium hydroxide and sulfuric acid that cannot be recovered and recycled, low crystallization yield.

An alternative process is the precipitation with ammonia. In this process, the pH of the fermentation broth is controlled by adding an ammonia-based solution, leading to the generation of diammonium succinate. Diammonium succinate is converted into succinic acid and ammonium sulfate via treatment with ammonium bisulfate and/or sulfuric acid. The by-product ammonium sulfate can be cracked thermally into ammonia and ammonium bisulfate. The precipitated succinic acid is separated and purified after dissolution in methanol and re-crystallisation. The advantages of the integrated precipitation with ammonia are the lower amount of waste by-products and the possibility of recycling base and acid. The main disadvantage is the low selectivity of the precipitation with ammonia.

Electrodialysis is a well-known method in the food industry for the separation of citric acid from citric juices (Zang et al., 2011). Bipolar membrane electrodialysis has been employed for the separation of succinic acid from fermentation broths (Fu

et al., 2014). In this process, sodium hydroxide is used as pH-titrant during the fermentation, resulting in di-sodium succinate (Na_2SA) as fermentation product. After fermentation, the biomass is separated via a conventional electrodialysis membrane, allowing to accomplish both separation of the biomass and concentration of di-sodium succinate (Glassner et al., 1992). Subsequently, a chelation-step using an ion-exchange column is required to replace divalent cations with sodium ions. Disadvantages of electrodialysis are the high energy consumption, the material costs of the mem- branes and the low selectivity for succinic acid.

Reactive extraction is a promising process for the separation of carboxylic acids from fermentation broths (Kurzrock and Weuster-Botz, 2011). For the reactive extraction of succinic acid from an aqueous phase, long-chain aliphatic primary, secondary and tertiary amines have been proposed and it is considered as an effective and economical purification method in recent years because the process is operated at normal temperature and pressure (Yang et al., 1991).

Most DSP approaches for succinic acid are principally functional. However industrial realisation depends greatly on scalability, robustness, overall separation yield and costs (Kurzrock and Weuster-Botz, 2010).

One example is the production of succinic acid with the natural producer *Anaerobiospirillum succiniciproducens* in a cell-recycle bioreactor. Meynial-salles et al., (2008) developed a three stages continuous fermentation process for the production of succinic acid at high concentration, productivity and yield. To limit end-products inhibition on growth, an electrodialysis system was coupled to the cell recycle bioreactor to allow the continuous removal of succinate and acetate from the permeate.

Wang et al., (2014) described the development of a novel integrated system for lactic acid production by *A. succinogenes*. Fermentation and separation were integrated with the use of a microfiltration (MF) membrane, and lactic acid was recovered by resin adsorption following MF. The fermentation broth containing residual sugar and nutrients was then recycled back into the fermenter after lactic acid adsorption. This novel approach overcame the problem of product inhibition and extended the cell growth period from 41 h to 120 h.

However, whether these types of complex processes are applicable at industrial scale is still an open question.

5.3.2 Genetic engineering

The majority of metabolic engineering processes fail during the scale-up phase. One of the reasons is the exposure of the bio-catalyst to a variety of stresses. As a consequence, cells spend more energy for maintenance requirements, that leads to several consequences, including a change in metabolic activity, lower growth rate, lower viability and lower productivity. Strain robustness, which is the ability of the microorganism to withstand the production environment, is therefore another key factor determining whether a microbial process will be successful and industrially viable. To this regard, the improvement of strain robustness against stress is an emerging research field of great interest, to move a step further in making microbial organic acid production processes become industrially viable.

Among the variety of available tools to induce genetic modifications and strain development, random mutagenesis and directed evolution are the only two approaches that have been applied in *A. succinogenes* to enhance the production of succinic acid and/or to reduce the formation of by-products (Zheng et al., 2013). Although suitable shuttle vectors have been constructed and were successful in homologous gene expression in *A. succinogenes* (Jang et al., 2007) there are no

focused metabolic engineering studies targeting the improvement of succinic acid production.

A possible solution for *A. succinogenes* would be the enhancement of succinic acid titer and yield by gene amplification of the pathways that lead to the desired product or deletion of genes that control pathways leading to the formation of other products. Genes that take part in the reductive pathway of the TCA cycle (PEPCK, MDH, Fm and Fr) could be amplified or genes responsible for by-product formation (AK, PFL) could be knocked out in order to favor the fluxes to the C4 pathway.

The following metabolic engineering strategies could be elaborated in future studies to enhance succinic acid production by *A. succinogenes*: i) increasing tolerance to product, ii) removing negative regulatory circuits limiting overproduction, iii) rerouting fluxes to optimize cofactor and/or precursor availability, and iv) optimizing metabolic fluxes towards product formation.

5.3.3 Biorefinery approach for SA

The biorefinery approach is a process strategy that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass (Cherubini, 2010). In a biorefinery, biomass is defined as any organic matter that is available on a renewable basis and this includes energy crops, agricultural and forestry residues, animal wastes and other organic residues. Biorefinery development has two strategic goals: i) the shift from imported petroleum in favour of renewable domestic raw materials (an energy goal); ii) the establishment of a robust biobased industry (an economic goal).

Succinic acid is one of the most promising value added bio-products that can be produced from different kind of renewable materials, in a biorefinery perspective. Bio-based succinate production facilities should be built in the same geographic area where the feedstock (e.g., corn or agricultural waste) is produced in order to minimize the transportation costs. Further cost-reduction could come from the co-production of high-value products. As an example, succinic acid was suggested as a coproduct of industrial ethanol fermentations (Lynd et al., 2002). A process more focused on large-scale succinate production is to produce ethanol and succinate in separate fermentations but at the same facility. This process would allow CO_2 waste from the ethanol fermentation to be used as substrate for succinate production (Zeikus et al., 1999). Figure 5.1 presents a potential biorefinery concepts for simultaneous production of succinic acid and ethanol.

Kuglarz et al., (2015) evaluated to use hemp for integrated biofuels and biochemical production. In particular, they evaluated two types of pretreatment (dilute-acid and alkaline oxidative method) for the combined bioethanol and succinic acid production. The acid-based pretreatment of hemp resulted to be superior to alkaline oxidative method. The mass balance calculations showed that 149 kg of EtOH and 115 kg of succinic acid can be obtained per 1 ton of dry hemp.

For some cases, a two-steps biorefinery approach has been suggested. This approach includes the use of an unexpensive carbon source to produce a stream of nutrients that is subsequently feed into a second fermentation step to be converted in the desired product.

This two-steps approach has been proposed by Du et al., (2007) for succinic acid production. In their work, wheat flour was converted into a microbial sugars-rich feedstock by a fungal strain. In the second stage, the feedstock is converted into succinic acid by *Actinobacillus succinogenes.* The wheat hydrolysate can substitute for refined glucose, and a fungal autolysate is a substitute for yeast extract.

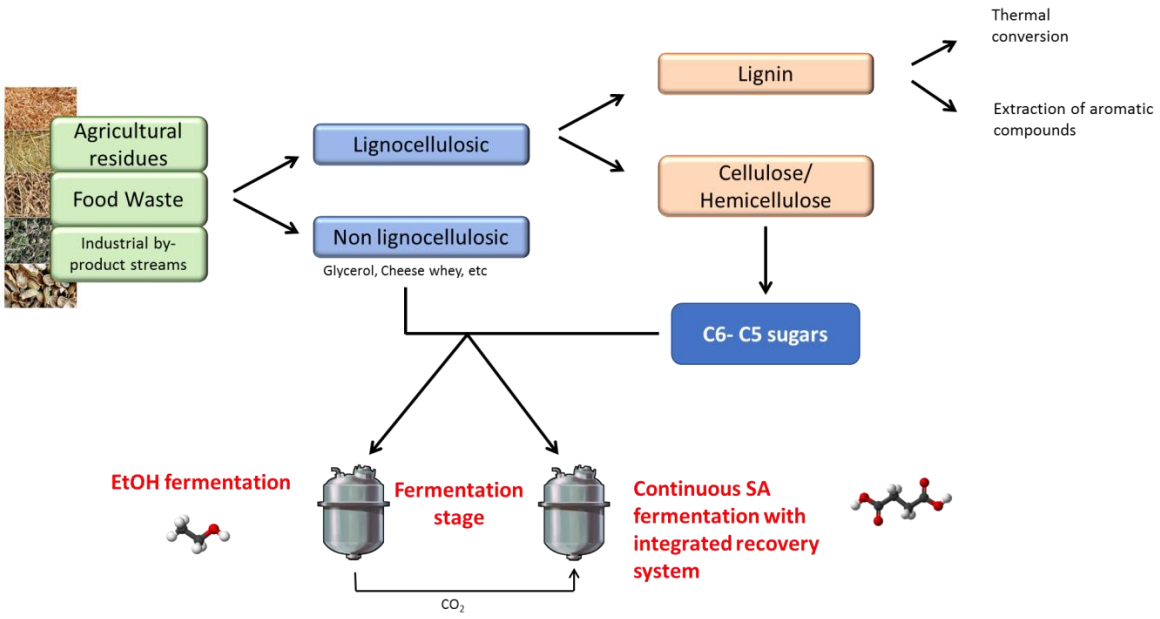

***Figure 5.1**: Integrated biorefinery for the simultaneous production of ethanol and succininc acid from renewable resources.*

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Raganati F., Procentese A., **Ferone M.**, Olivieri G., Russo M.E., Salatino P. Marzocchella A., Continuous glucose fermentation by *Clostridium acetobutylicum* – kinetics issues under acidogenesis and solventogenesis conditions. Submitted to International Scientific Journal.

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Ferone M , Raganati F, Olivieri G, Salatino P ,Marzocchella A, Succinic acid production by fermentation of sugars representative of lignocellulosic hydrolysate, 3rd European Congress of Applied Biotechnology (ECCE10+ECAB3+EPIC5),September 26th-October 1st 2015 , Nice (France). Oral presentation

Ferone M , Raganati F, Olivieri G, Salatino P ,Marzocchella A, Succinic acid production from hexoses and pentoses by batch fermentation of *Actinobacillus succinogenes*, 5th International Conference on Industrial Biotechnology, April 12-16th 2016, Bologna (Italy). Oral presentation

Ferone M , Raganati F, Olivieri G, Marzocchella A, Salatino P. Continuous production of succinic acid by fermentation of *Actinobacillus succinogenes*, 17th Eupopean Congress on Biotechnology, July 3rd -6th 2016, Krakow (Poland). Poster contribution

Carpine R., **Ferone M**., Gifuni I., Marzocchella A., S. Niglio, Olivieri G., Peirce S., Procentese A., Raganati F., Russo M.E., Salatino P., Salemme L.. Intensification systems in bioconversion processes. GRICU 2016 Congress "Gli orizzonti 2020 dell'Ingegneria Chimica". 12th - 14th Sepember 2016, Anacapri (Naples, Italy). Oral presentation

Karlsson E, **Ferone M**, Mapelli V, Olsson L .Tolerance to adipic acid for future microbial cell factories: Candida viswanathii vs. Saccharomyces cerevisiae, Accepted for poster 13th International Conference on Renewable Resources and Biorefineries, June 7th - 9th 2017 Wroclaw (Poland). Poster contribution

Ferone M , Olivieri G, Salatino P ,Marzocchella A Succinic acid production by fermentation of *Actinobacillus succinogenes* in a continuous packed bed bioreactor, 10th Word Congress of Chemical Engineering (WCCE10+ECCE11+ECAB4), 1st -5th October 2017 , Barcelona (Spain). Oral presentation

COLLABORATION WITH FOREIGN RESEARCH INSTITUTION

January – July 2017: Research activity at Chalmers University of Technology, Department of Biology and Biological Engineering, Industrial Biotechnology group on the issue: "**Development of a microbial cell factory for adipic acid production"**. Supervisor: Lisbeth Olsson

COURSE AND SEMINARS

Attended course

- "Structuring and Writing Manuscripts for Publication in Scholarly Journals: Adding Value to the Scientific Literature" – Prof. Barnett Parker, 15-17/06/2015
- "Impianti dell'industria di processo" parte del corso – Prof. Antonio Marzocchella- November-December 2014
- Summer School: "Towards a Bio-based Economy: Science, Innovation, Economics, Education". 4-8/09/2017. University of Milano-Bicocca
- PhD course: "Industrial Biotechnology for Lignocellulose Based processes". 22-27/10/2017. Chalmers University of Technology

Seminaries

1. **Carlos Regalando** "Edible films and coatings to increase shelf life of fresh foods", 09/07/2015
2. **Blanca Garcia** "Microbial study of Listeria monocytogenes biofilms", 09/07/2015
3. **Rafaele Porta** "Le transglutaminasi: dalle poliammine alle bioplastiche", 30/06/2015

4. **Peter Gotz** “Microbial production of polysaccharides as feed additives”, 14/04/2015
5. **Alfredo Ronca** “Bioactive composite scaffolds for bone regeneration: from the process to the biological validation”, 25/03/2015
6. **Piero Salatino** “Fluidizzazione di solidi granulari e mobilità di flussi piroclastici densi”, 29/01/2015
7. **Angelo Fierro** “Il Grafalloon del bioetanolo di seconda generazione: il caso studio per la regione Campania”, 11/02/2015
8. **Roberto Lauri** “Aspetti di sicurezza relativi a processi industriali finalizzati alla produzione di biocombustibili e bioplastiche”, 28/012015
9. “Biotecnologie per lo sviluppo sostenibile: applicazioni e sicurezza” - Prof. Biancamaria Pietrangeli, 28/01/2015
10. **Franco Terlizzese** “La strategia italiana per l’utilizzo delle materie prime e il loro impatto ambientale: idrocarburi e geotermia”, 17/11/2015
11. **Thierry Tron** “ Functionalized and artificial enzymes: new bio-derived catalysts , 14/01/2016
12. **Angharad Gatehouse** “Biopesticides which target voltage-gated ion channels: efficacy and biosafety ,14/01/2016
13. **Barbara Sherwood Lollar** “New development in isotopic investigation of source and fateoh halogenated hydrocarbon compounds, 17/05/2016
14. **Lars Rehmann** “Fermentative butanol production form unconventional resource,14/06/2016
15. **Angelo Fontana** “Research and exploitation of marine genetic resources: from ecophysiology to biotechnology, 13/07/2016
16. **Bogdan Bjola, Michele Maremonti** “La protezione brevettuale: opportunità, procedure, casi di studio, 30/09/2016
17. **Tomas Morosinotto**: “Algae: metabolic engineering for the sustainable production of bio-commodities”, 06/12/16.
18. **Raffaele Scoccianti**: “Effective comunication in industry (and tips for building a strong CV)”, 23/11/2016
19. **Edgardo Filippone**: “ Dalle piante alle microalghe, il mondo biotech si tinge di verde”, 26/10/2016
20. **Dionysio Dionysiou**: “ treatment of cyanotoxins and contaminants of emerging concern in water using advanced oxidation processes”, 27/10/2016
21. **Tomaso Zambelli**: “FluidFM for single cell manipulation”, 18/10/2016
22. **Tomaso Zambelli: “**Development of FluidFM and its application for 2D patterning as well as 3D microprinting”, 18/10/2016

APPENDIX

The activities reported in this section were carried out during a research stay at Chalmers University of Technology (Göteborg, Sweden), in the Industrial Biotechnology division, under the supervision of Prof. Lisbeth Olsson.

These activities aimed at the development of a microbial cell factory for adipic acid production. In particular the two main objective were: i) identification and physiology study of an adipic acid tolerant host suitable for industrial production ii) identification an native (or engineered) enzyme able to perform one of the enzymatic step of the metabolic pathway proposed to produce adipic acid.

This research is part of a wide collaborative project financed by Formas, the Swedish research council for sustainable development, and aims at using Swedish renewable sources for the establishment of a biorefinery (Formas Diarienummer 213-2013-78; www.biobuf.se).

This research will help in increasing the level of knowledge available nowadays on the microbial production of organic acids - as succinic and adipic acid - accelerating the way towards their biobased production from renewable resource.

A.1 Tolerance to adipic acid for future microbial cell factories: *Candida viswanathii* vs. *Saccharomyces cerevisiae.*

Mariateresa Ferone[1,2], Emma Karlsson[2], Valeria Mapelli[2], Lisbeth Olsson[2]

[1] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale – Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

[2] Chalmers University of Technology, Department of Biology and Biological Engineering, Division of Industrial Biotechnology, SE-412 96 Göteborg, Sweden

Background

Over the last decade, there has been a growing interest in producing chemicals from renewable resources because of the depletion of oil resources and the economic and environmental issues associated with petroleum-based chemicals. The processes of biochemicals production from side streams or renewable resources are also interesting as potential solutions to remediation problems, a critical issue for industries from both the technical and economic point of view (Alonso et al., 2014).

Adipic acid or hexanedioic acid ($C_6H_{10}O_4$, MW 146.14) is an aliphatic di-carboxylic acid currently produced from oil. From an industrial perspective, it is one of the most important bulk chemicals with a world production of about 3 million tons per year (de Jond et al., 2011).

The primary application of adipic acid is in the chemical production of nylon-6,6 polyamide, which represents 61% of global consumption of the acid. Adipic acid is also used to produce polyurethanes, as a reactant to form plasticizers, lubricant

components and polyester polyols. Small, but significant amounts of adipic acid are used as a food ingredient (E355) in gelatins, desserts and other foods that require acidulation (Musser, 2005).

Currently, most of the commercially available adipic acid is produced by a chemical process which starts from benzene. Benzene is first reduced to cyclohexane, which is then converted to a mixture of cyclohexanone and cyclohexanol by two successive oxidation steps. This mixture is further reacted to adipic acid with nitric acid and air using copper or vanadium as catalyst. Adipic acid is also made by the hydrocyanation of butadiene, followed by hydroisomerization to adiponitrile, which then is hydrolyzed (Park and Goroff, 1993). A byproduct of this process, however, is N_2O, a gas commonly thought to be a contributor to global warming and ozone depletion (Sato et al., 1998). It is estimated that about 10% of the worldwide anthropogenic N_2O emission originates from the production of adipic acid (Allini et al., 2007). The development of an environment friendly chemical production of adipic acid needs a strong reduction of nitrous oxide emission, for example by N_2O-decomposition in industrial conditions.

The bio-based production of adipic acid, particularly from renewable resources, would be an attractive alternative that would address environmental concerns regarding the harsh chemicals used in the classical production route and a complete substitution of the chemical production route has been estimated to prevent the annual production of 7.9 tons of greenhouse gases (de Jond et al., 2011).

In order to be a good host for an industrial application, the microorganisms should tolerate high titres of the acid, i.e., in the range 50–100 g/L (Wang et al. 2016, Warnecke and Gill, 2005). In addition, the microorganism should also preferably tolerate low pH, as the overall cost of processing at low pH is reduced, due to the lower amount of base required, and less complex downstream purification (Tsuge et al. 2016).

It has been demonstrated that microbial production of adipic acid is possible (Yu et al., 2014), but the yields and titres are too low for commercialisation. The reason for this could be linked to the specific metabolic pathway employed. However, the low yields and titres of microbially produced adipic acid so far could be due to acid stress and poor cellular tolerance to adipic acid itself. Product toxicity has been identified as one of the primary challenges in developing a bioprocess for organic acid production (Warnecke and Gill, 2005, Deng and Mao, 2015, Beardslee and Picataggio, 2012). Karlsson et al., (2017) have reported a screening study to investigate which microorganism(s) have the potential for use in a microbial cell factory for the production of adipic acid, based on their tolerance to adipic acid. The yeast species *C. viswanathii* (strain NCYC 997) exhibited the highest tolerance to adipic acid and was almost unaffected by the presence of even the highest adipic acid concentration tested (650 mM adipic acid). In the present work, *C. viswanathii* was further studied to have more insights into its metabolism when it grow in presence of adipic acid. The well-known yeast *Saccharomyces cerevisiae* was also included in this study as comparison.

Materials and Methods

Strains and cultivation media

The microorganisms included in this study were the yeasts *Saccharomyces cerevisiae* CEN.PK 113-7D and *Candida viswanathii* NCYC 997.

Yeasts were cultivated in minimal medium, containing: 20 g/ L glucose, 5 g /L $(NH_4)_2SO_4$, 3 g /L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 mL /L vitamin solution, 1 mL /L trace element solution. Vitamin solution and trace element solution were prepared as previously described (Verduyn et al., 1992). In addition, 0.15 mL/L silicone antifoam was added to prevent excessive foaming. Where required, adipic acid was also added to the media at pH of 5. Adipic acid concentrations investigated were: 0, 96 and 650mM.

Fermentation

Yeast was precultured in defined medium (as described above) until the exponential growth phase. The inoculum was cultivated in Erlenmeyer flasks incubated at 30 °C and 200 rpm. A volume of inoculum that resulted in an OD_{600} of 0.1 was added to the main cultivation. The main cultivations were carried out in DASGIP parallel bioreactor systems comprising of four units, each holding four SR0700ODLS vessels (DASGIP, Jülich, Germany). The culture volume was 600 ml and the fermentors were pre-conditioned overnight at pH 5. Aeration was set to 1 vvm at an impeller speed at 400 rpm. A feedback loop was created between the impeller speed and the dissolved oxygen probe signal to maintain aeration above 40 % of oxygen saturation. Each cultivation condition was performed in duplicate. During the cultivation process, CO_2 production or O_2 consumption was measured continuously using an off-gas analyzer.

Analytical methods

Analysis of ethanol, glycerol, acetate and adipic acid from the cultivation was performed by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC unit (Thermo scientific, Dionex Corporation, Sunnyvale, USA) equipped with an Rezex ROA organic acid (Phenomenex)) column 300 mm long and 7.8 mm in diameter, which was packed with 9 µm particles. A column temperature of 80°C was used for analysis and 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 ml/min. A Shodex RI-101 RI detector and an Ultimate 3000 VWD 3100 variable wavelength ultraviolet detector coupled to the HPLC unit were used to quantify the metabolites.

Growth was followed by OD_{600} measurements using a Thermo Scientific GENESYS 20 Visible Spectrophotometer for measurement of the optical densities of the cultures. Determination of dry cell weight was performed in duplicate. 5 ml of the culture was filtered using pre-dried and weighed filter paper discs of 0.45 µm pore size (Sartorius Stedim Biotech, Goettingen, Germany) on a water tap vacuum filter unit (Sartorius Stedim Biotech, Goettingen, Germany). The filter paper discs were dried in a microwave at 120 W for 15 min, weighed again and the biomass was determined from the difference.

Extraction of intracellular ATP, ADP, AMP

Cellular content of adenosine nucleotides was determined as described previously (Ask et al., 2013). In short, samples (two of 5 mL each) were quenched in 25 mL pure methanol at - 40 °C. Cells were harvested by centrifugation of the samples at 4000 g for 5 min at -10 °C. Extraction of cellular ATP, ADP, and AMP was performed by incubation of cell pellets with 0.5 mL 0.52 M trichloroacetic acid containing 17 mM EDTA on ice for 15 min. After that, 400 µL supernatant obtained after centrifugation of the mixture at 13 000 g for 10 min was neutralized by 116 µL 2 M Tris–base (pH 10.8).

Results and discussion

The experiments were carried out in batch cultures with 20 g/L glucose as carbon source supplemented with adipic acid under aerobic conditions. at pH 5.0. For comparison, yeast cells grown without adipic acid are referred to as the control.

Figure A.1.1 shows the growth curve of *S. cerevisiae* (Figure A.1.1a) and *C. viswanathii* (Figure A.1.1 b) in the presence of different concentration of adipic acid, compared to their relative controls. In the control, *S. cerevisiae* CEN.PK 113-7D showed the typical two phase growth curve when grown in normal defined medium with glucose as carbon source. Yeast cells first grew on glucose producing mainly biomass and ethanol (respiro-fermentative phase), and after all glucose had been consumed, the cells started to grow on the acetate, glycerol and ethanol produced during the process (respiratory phase). No apparent initial lag phase was observed for yeast cells grown on glucose, and there was the typical short lag phase before cells shifted to the fully respiratory metabolism. During respiro-fermentation, *S. cerevisiae* cells were able to grow in the presence of 96 mM adipic acid (Fig. A.1.1a), but an initial lag phase was observed. After the diauxic shift, when yeast metabolism changed from respiro-fermentative to fully respiratory, respiratory growth was inhibited at 96 mM adipic acid.

In the control, *C. viswanathii* showed the typical growth curve of a fully respiring microorganism growing on glucose. In a previous study (Karlsson et al., 2017), it has been suggested that this yeast has a primarily respiratory metabolism, as has been proposed for the closely related *Candida tropicalis* (Mishra et al. 2016).

In contrast to the growth behaviour exhibited by *S. cerevisiae*, *C. viswanathii* was able to grow even in the presence of the highest concentrations of adipic acid (650 mM) tested. Unfortunately, a contamination become visible after 40 hours during the growth experiment with *S. cerevisiae* in the presence of 650 mM of adipic acid and at that time point *S.cerevisiae* did not exhibit any growth.

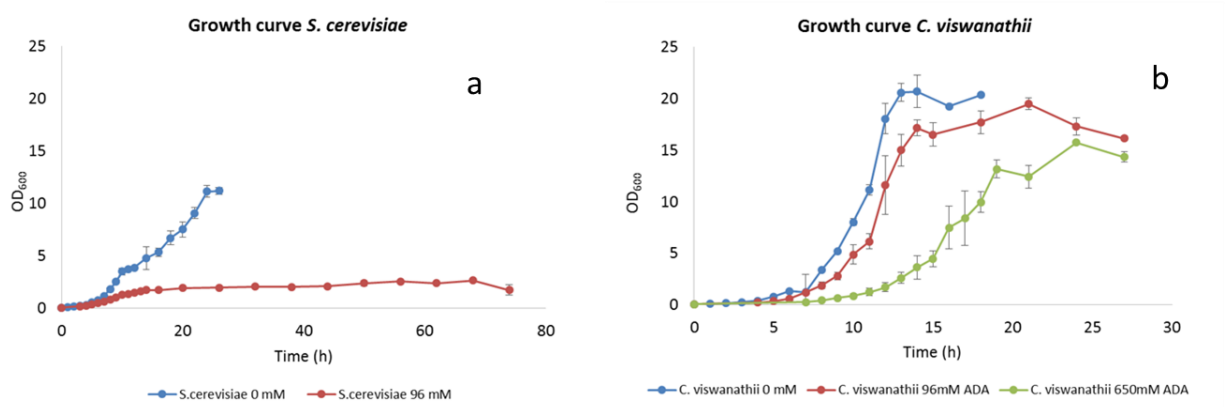

***Figure A.1.1**: S. cerevisiae and C. viswanathii growth curves in presence of different concentration of adipic acid*

In Figure A.1.2a the values of the specific maximum growth rate (μ_{max}) is shown, and Figure A.1.2b shows the maximum concentration of the metabolites produced from glucose for both the yeasts.

C. viswanathii specific growth rate was not affected in the presence of 96mM of adipic acid whereas it was reduced by 25% in the presence of 650mM of acid; *S. cerevisiae* specific growth rate was reduced by 35% in presence of the lowest concentration of adipic acid used.

It is also interesting to note, as shown in Figure A.1.2b, that *C. viswanathii* produced glycerol when it grows in the presence of adipic acid and that the concentration of glycerol produced increased with increasing adipic acid concentration in the medium. Glycerol is known to be a metabolite produced to cope with osmotic stress in *S. cerevisiae* (Albertyn et al, 1994). In addition, glycerol synthesis in *S. cerevisiae* is used to regenerate NAD $^{+}$, in particular when the route for ethanol synthesis is inhibited.

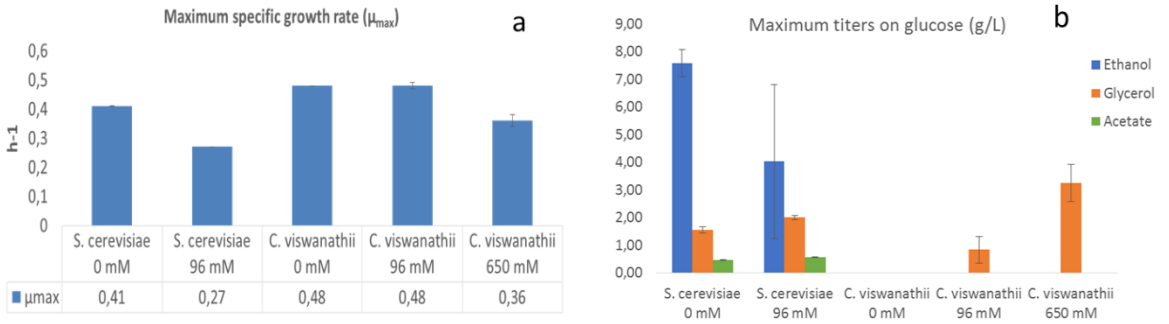

***Figure A.1.2:** a) S. cerevisiae e C. viswanathii maximum specific growth rate in presence of adipic acid e b) maximin metabolite concentration produced from glucose.*

In this study, the extracellular concentrations of adipic acid did not vary during growth, indicating that it was not consumend by the yeasts. However, the technique

used for the quantitative measurament (HPLC) of adipic acid did not allow do ascertain if the there is any diffusion of the acid across the membrane.

Verduyn et al. (1992) showed that benzoic acid enters the cells mainly via passive diffusion of the undissociated form of the acid; hence it can be hypothised that the same mechanism applies for adipic acid. Further experiments (i.e. experiments with isotope labelled adipic acid) will be necessary to prove the actual mode of inflow and action of adipic acid on the cellular physiology leading to the effect we observed on the specific growth rate.

Under optimal conditions, ATP generated is used for cell formation and maintenance. When cells encounter acid stress, there is an additional need for ATP by the cellular H^+ pumps to remove H^+ ions from the cytosol to maintain the intracellular pH at physiological values (Liu et al., 2015). This additional need for ATP re-directs the ATP formed away from cell mass formation and possibly also maintenance. During these tests, extra samples were withdrawn in order to extract energy molecules (ATP, ADP, AMP), aiming at the evaluation of how intracellular energy content varies when the two yeasts grow in the presence of adipic acid. Preliminary data were generated and a protocol for the quantitative analysis of the extracted molecules is currently under development.

Under acid stress conditions, the yeast cell induces a common set of functional changes as a global response to stress. These changes include, on one hand, reduction in activities linked with cell proliferation and protein synthesis, anabolic pathways and other processes associated with high energy expense, and, on the other, increase in activities related to protection and repair of damage of different molecules (DNA, proteins and lipids) and cellular structures. This change in the metabolic characteristic of the cells implies a different gene expression (Dong et al., 2017).

During the fermentations, additional samples were withdrawn to perform qPCR experiments for quantitative analysis of genes expressed under stress conditions. However, the analysis of specific target transcripts has not been performed yet.

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A.2 Fumarate reductase as a potential enzyme to catalyse an enzymatic step for adipic acid biosinthesys

Mariateresa Ferone[1,2], Emma Karlsson[2], Valeria Mapelli[2], Lisbeth Olsson[2]

[1] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale – Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125 Napoli – Italy

[2] Chalmers University of Technology, Department of Biology and Biological Engineering, Division of Industrial Biotechnology – Industrial Biotechnology, SE-412 96 Göteborg, Sweden

Background

Among various commodity chemicals, adipic acid (C6) is a high-volume dicarboxylic acid used as a chemical intermediate in the commercial manufacture of nylon 6,6, thermoplastic polyurethane resins, plasticizers, adhesives and synthetic lubricants. Annually, more than 2.6 million tons of adipic acid are produced through a chemical process using benzene derived from fossil fuels (Polen et al., 2013). Adipic acid is currently produced via a petrochemical process, starting from benzene, that goes through an oxidation process with nitric acid and air. During this process, N_2, NO,NO_2 and N_2O are formed, with nitrous oxide as main byproduct. These NOx emissions are a major environmental concern (Alini et al., 2007). Several attempts to produce adipic acid, or more often, precursors of adipic acid, by fermentation using selected or engineered microbial strains have been published.

An example is the biosynthetic pathway for the conversion of glucose to cis,cis-muconic acid demonstrated in recombinant *E. coli* by Niu and colleagues in 2002 (Niu et al., 2002). Their pathway consisted of genes from *Acinetobacter sp.* and *Klebsiella pneumonia* for the conversion of glucose to catechol (1,2-dihydroxybenzene) through the shikimate pathway, followed by the oxygenation of catechol to cis,cis-muconic acid. The authors demonstrated that following the purification of cis,cis-muconic acid, the product could be chemically converted to adipic acid by a hydrogenation reaction (10% Pt/C, H_2, 3400 kPa, 25°C).

More recently, the conversion of biomass feed stock directly to adipic acid in a single process has received much attention in the biotechnology sector. A total of eight metabolic pathway have been proposed in four different patents (Bugard et al., 2010, Picataggio and Beardslee, 2011, Raemakers et al., 2010, Baynes and Geremia, 2010).

Although several patents have been filed for the production of adipic acid, pathways for its synthesis are not known to exist naturally in any organism, and efforts are necessary to discover novel enzymes for completion of metabolic pathways leading to adipic acid.

Among the pathways proposed, one is based on the use of the amino acid lysine as a starting point to synthesize adipate. This pathway (Fig. A.2.1) for adipate biosynthesis by means of a lysine degradation pathway has been described by Burgard et al., (2010). The authors describe a pathway wherein lysine is degraded to

6-amino-hex-2-enoate by a carbon-nitrogen lyase, and further to 6-aminocaproic acid by an oxidoreductase. Following this step, 6-aminocaproate may undergo a transaminase-catalyzed reaction to produce adipate semialdehyde, which may be oxidized by an alcohol dehydrogenase to produce adipate.

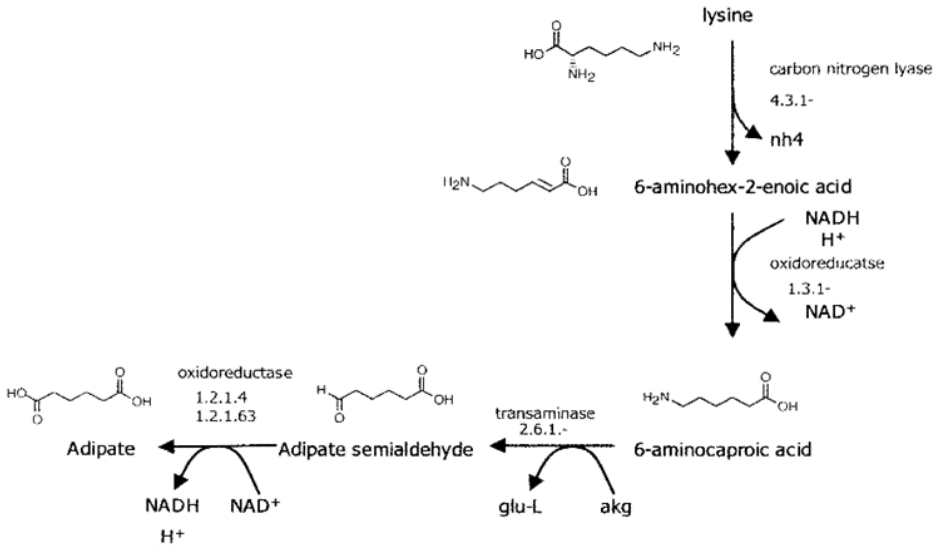

Figure A.2.1*: One of the possible metabolic pathway for the production of adipic acid starting form lysine; the figure includes the class of enzymes, the EC number and the cofactors needed.*

The second step of the proposed metabolic pathway to produce adipic acid consists of the reduction of the unsaturated bond in α-β position with respect to the carboxylic group. The aim of the present work is to investigate if fumarate reductases can perform the targeted reaction. The fumarate reductases are an interesting enzyme group since they can reduce α-, β– unsaturated bond of a carboxylic group. Fumarate reductase is often used as terminal oxidant for respiration when oxygen is absent (Reid et al., 2000).

Two fumarate reductases were chosen for this study: a fumarate reductase from the baker yeast *Saccharomyces cerevisiae* (Frd1, UniProt Entry: P32614) and the fumarate reductase flavoprotein subunit (SO_0970, UniProt Entry: P83223) from the bacterium *Shewanella oneidensis*. These enzymes were selected since they are expressed as soluble enzymes whereas many other fumarate reductases are membrane bound, often containing Fe/S clusters (Cecchini et al., 2002). These enzymes were expressed as recombinant proteins in *E.coli* and an enzymatic assay to test their activity towards the target substrates was set up.

Materials and Methods

Fumarate reductases expression and purification

To perform the in vitro assays, the enzyme were overproduced in *E*.coli Rosetta (DE3) (Thermo Fisher Scientific) carrying the plasmid pET28 which contains the sequence of *Frd1 or SO-0970.* The sequences of both the enzymes were optimized for the expression in *E.coli. Frd1 and SO-0970* were both expressed with a N-terminal His-tag.

The transformed bacterial strain was inoculated in 10mL LB medium containing ampicillin (100µg/mL) and kanamycin (50µg/mL) overnight at 37° C. The expression of the enzymes was induced by autoinduction method. The auto-induction method of protein expression in *E. coli* is based on diauxic growth resulting from dynamic function of *lac* operon regulatory elements (*lacO* and LacI) in mixtures of glucose, glycerol and lactose (Studier, 2005). The autoinduction media is prepared by adding to LB medium the following components: 0,05 % Glucose, 0,5 % Glycerol, 0,2 % Lactose, 25 mM KH_2PO_4, 25 mM Na_2HPO_4, 2 mM $MgSO_4$

This preculture was the used to inoculate 1L fresh LB medium containing ampicillin (100µg/mL) and kanamycin (50µg/mL) grown at 37°C After 24 hours of autoinduction, the bacterial culture was harvested by centrifugation (5000 rpm, 15 min, 4°C) . Cell pellets were resuspended in 30 ml of lysis buffer (50mM Na_2HPO_4, 300mM NaCl, 20mM imidazole), 30µL of lysozyme (10mg/ml) was added and cells were incubated for 4 hours at 4°C. Another step of centrifugation was performed to collect the soluble protein (10000 rpm, 4°C, 30 min). The resulting supernatant was then loaded onto a HisTrap excel 1ml column and an Äkta Purifier chromatographic system (both the column and the purification system from GE Healthcare, Life Science). The enzymes were eluted using a buffer containing 500 mM imidazole.

To determine the protein concentration the Bradford method was used, with bovine serum albumin as a standard. The purity of the enzyme after this purification step was checked by SDS gel.

Enzymatic activity measurement: assay set-up

The enzyme activity was determined spectrophotometrically by measuring the increase in the absorbance of FAD^+ at 440 nm. The reaction was carried out under anaerobic conditions to avoid the spontaneous reoxidation of $FADH_2$ to FAD^+ by oxygen. The purified enzymes were preincubated in a 96 well plate in buffer (potassium phosphate buffer 100mM pH 7.4) and 1 mM cofactor; sodium hydroxide was added to reduce FAD+ to $FADH_2$. Then 50mM of substrate (fumarate) were added to start the reaction. All the solutions (except for the enzymes and the cofactor) were previously degassed by sparging with N_2. The reaction mixture was prepared inside the anaerobic hood, then the plate is placed in the anerobic platereader with $O_2 < 0.1\%$ (CLARIOstar, BMG LabTech).

The anaerobic assays were performed at the Department of Molecular and Clinical medicine, University of Gothenburg.

Site- directed mutagenesis

The plasmid pET28 used in this study was propagated in DH5α *E. coli* cells and it was prepared using GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific). Phusion High Fidelity DNA polymerase and *DpnI* restriction enzyme are provided by Fermentas (Thermo Fisher Scientific, USA).

PCR was carried out in a 50µL mixture using 20 ng of template plasmid DNA (pET28_Frd1 or pET28_SO_0970), 125ng of each primer, 250µmol of dNTPs, 1 Unit of Phusion High Fidelity DNA polymerase in 1X Phusion HF reaction buffer. The PCR cycles were programmed as follow: 98°C for 1 minutes to denature the template DNA;18 cycles at 98 °C for 30 sec, optimal melting temperature (T_M) for 30 sec,

elongation at 72°C for 6 min; final elongation step at 72 °C for 10 min; cool down at 4 °C for 10 min.

The primers used for mutagenesis are shown in the Table A.2.1.

The PCR products were treated with 1µL of *DpnI* at 37°C for 1 hours and then 10 µl of each PCR reactions was analyzed by agarose gel electrophoresis. PCR products were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific) and used to transform DH5α *E. coli* cells for plasmid propagation.

Chemically competent *E. coil* cells were transformed by heat shock. 10µL of the ligation mix were added to the competent cells and thawed in ice for 30 minutes. For the heat shock the tube was then placed at 42°C for 1,5 minutes and put back in ice for 2 minutes. After transformation, one hour incubation in 900 µL of SOC medium was performed (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10 mM NaCl, 2.5mM KCl). Cells were plated on plates containing the appropriate antibiotics and incubated O/N at 37°C. Positive clones were cultured overnight in LB medium supplemented with the appropriate antibiotics (4 ml cultures), plasmids were isolated and final vectors were checked by sequencing. Sequencing of DNA fragments was performed at Eurofins Genomics with T7 and T7_term primers, which allow the sequencing of genes inserted in pET28 vectors.

Table A.2.1*: primers used for mutagenesis*

Oligonucleotide	Sequence 5'-3' (*)	T_M (**)	Description
M260A_R	CGCTCGCGCCACCCGCACGACCCACGTCAGTC	85.0	Mutagenic primer for SO_0979
M260A_F	GACTGACGTGGGTCGTGCGGGTGGCGCGAGCG	85.0	Mutagenic primer for SO_0979
M399A_R	CTGCCTCGGTGATCGCCACGCCACCCGCCGG	85.0	Mutagenic primer for SO_0979
M399A_F	CCGGCGGGTGGCGTGGCGATCACCGAGGCAG	85.0	Mutagenic primer for SO_0979
L115A_R	GCCACAGAGTGGCCACCCGCTTGAGCCAGCAGG	83.7	Mutagenic primer for Frd1
L115A_F	CCTGCTGGCTCAAGCGGGTGGCCACTCTGTGGC	83.7	Mutagenic primer for Frd1
L266A_R	GCAGGGATTCGGCAGCCGCGAATTTCCAGCTTGAG	83.3	Mutagenic primer for Frd1
L266A_F	CTCAAGCTGGAAATTCGCGGCTGCCGAATCCCTGC	83.3	Mutagenic primer for Frd1

***** Mutated nucleotides are underlined

****** T_M was calculated according to the formula: $T_M = 81{,}5 + 0{,}41(\%GC) - 675/N - \%mismatch$

Results

Frd1 and SO_0970 expression and purification

After 24 hours induction at 37 °C, the expression of Frd1 and SO_0970 in Rosetta (DE3) was examined. The crude lysate was separated into soluble and insoluble fractions by centrifugation and both fractions were analyzed by SDS–PAGE.

Since the Frd1 and SO_0970 genes are fused with a His-tag, the purification of the enzymes was performed by a chromatographic step using IMAC (Immobilized Metal Affinity Chromatography) with Ni- NTA resin material. Frd1 and SO_0970 have a native molecular weight of ~50 kDa and ~62 kDa, respectively. As shown by SDS-gel electrophoresis (Fig.A.2.2), with a single chromatographic step, the purification of the enzyme was partially achieved and bands of the expected molecular size are found in the collected fractions.

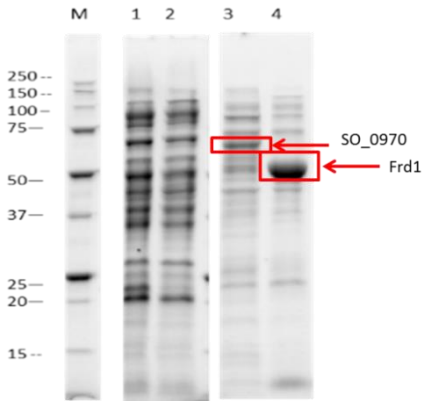

***Figure A.2.2**: SDS-page analysis of purified enzymes; column M: molecular weight marker (Fermentas, Thermo Fisher Scientific, USA); column 1: flowthrough for O_0970; column 2: flowthrough for Frd1; column 3: SO_0970 after purification; column 4: Frd1 after purification*

The graph in Figure A.2.3 shows the results of a preliminary activity assay of the fumarate reductase from *S. cerevisiae* (Frd1). In the presence of the enzyme, the absorbance increases to a greater extent than in the negative controls (in the absence of enzyme or substrate). This indicates the presence of enzymatic activity.

Unfortunately, the results of these essays are only partial and it will be necessary to continue this collaboration in the future in order to characterize such enzymes.

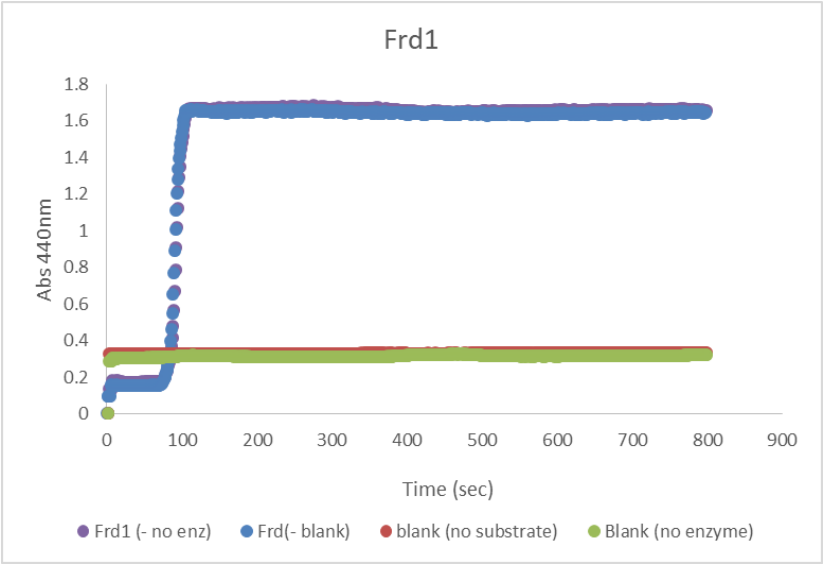

***Figure A.2.3**: preliminary results of enzymatic assay for Frd1; the reaction was monitored by following the absorbance of FAD^+ at 440nm in anaerobic conditions.*

Site - directed mutagenesis

Molecular docking experiments (data not shown) carried out on the enzymes of interest showed how the substrate must be placed within the active site so that the

reaction can take place and that the target substrate is in the correct position to allow the catalysis. For this reason, amino acid substitutions have been suggested to enlarge the active site to allow the target substrate (6-amino-es-2-enoic acid) to be properly positioned. Suggested mutation were: for Frd1: L115A and L266A; for SO_0979: M260A and M399A.

Figure A.2.4 shows the agarose gel electrophoresis of the PCR reactions products obtained.

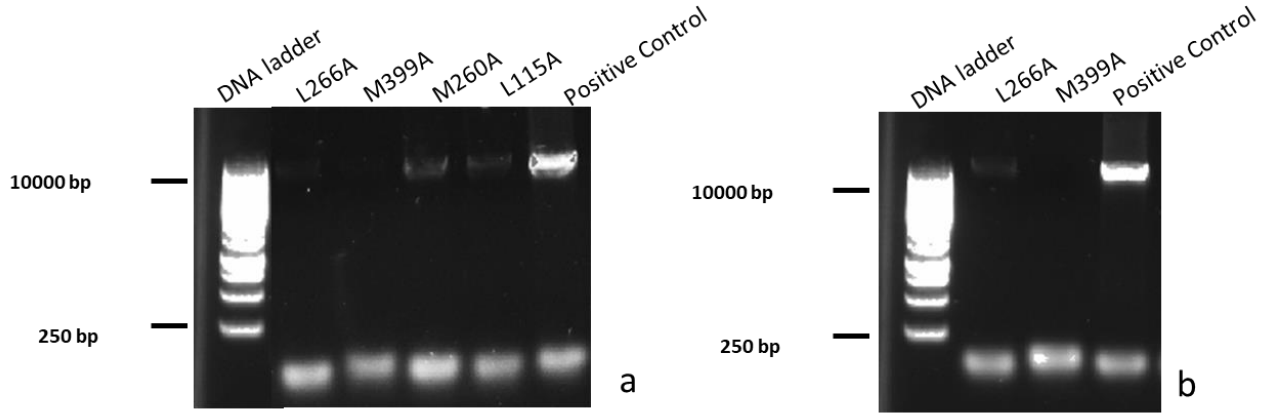

Figure A.2.4: *PCR amplification for single-site mutagenesis. A) Agarose gel electrophoresis of the PCR reactions carried out with an annealing temperature of 58°C; B) Agarose gel electrophoresis of the PCR reactions carried out with an annealing temperature of 72°C.*

Amplification bands were obtained for L115A, L266A and M260A, while no amplification was obtained for M399A at both temperatures tested. The sequencing results confirmed that the correct mutation was obtained.

Conclusion and future perspectives

Two soluble fumarate reductases, Frd1 from *Saccharomyces cerevisiae* and SO_0970 from *Shewanella oneodensis*, were expressed as recombinant protein in *E. coli* and purified. To test the activity of these enzymes, a specific assay conducted under anaerobic conditions, was set up. The preliminary results of the enzymatic assays indicate that both the enzymes were active on their natural substrate – fumarate. However, more experiment is needed to elucidate the activity of the selected enzymes on the target substrates, 6-amino-hex-2-enoate.
Moreover, the mutated enzymes will also be expressed and their activity will be tested by means of the developed assay.
This work will help in accelerating the way towards the biobased production of adipic acid and also in increasing the level of knowledge available nowadays on these enzymes.

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